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ABEL'S
LABORATORY HANDBOOK OF BACTERIOLOGY

OXFORD: HORACE HART
PRINTER TO THE UNIVERSITY

Abel, Rudolf Valentin Ludwig. 1868-194

OXFORD MEDICAL PUBLICATIONS

ABEL'S LABORATORY HANDBOOK OF BACTERIOLOGY

TRANSLATED FROM THE TENTH
GERMAN EDITION

BY

M. H. GORDON, M.A., M.D. Oxon., B.Sc.

WITH ADDITIONS

BY

DR. A. C. HOUSTON, DR. T. J. HORDER
AND THE TRANSLATOR

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PREFACE TO THE ENGLISH TRANSLATION

Any one who has been at all in the position to follow for some time the evolution of medicine and natural science cannot fail to have noticed how the importance and the use of bacteriological knowledge has constantly been spreading. For the physician and the veterinary surgeon bacteriology is an indispensable aid towards the recognition of infectious diseases in men and animals. Only by the aid of a certain amount of bacteriological knowledge is he enabled to understand and apply the successful methods of serumtherapeutics, of protective inoculation, of the delicate modern serodiagnostic methods of investigation, which promise still greater results. The commissioned medical officer has absolute need of bacteriology in order to employ proper and efficient measures to ward off pestilence and to prevent the spread of infectious diseases. Indeed, one may say that without bacteriology hygiene would never have attained its present height. The chemist, the druggist, the engineer, and even many commercial men, for instance, those working in the production of beer and wine, in the industry of preserves and of foodstuffs require a certain amount of knowledge of bacteriology for their business. Thus it has become the subject of study in ever-increasing circles.

In writing the present pocket edition it has been the wish of the author to provide those studying bacteriology with a guide to practical laboratory work. It is not meant to take the place of a textbook or of personal instruction, but to serve as a supplement to the above, to give practical hints, and to collect in practical form those technical details which so easily slip the memory.

Its popularity in Germany, where since 1898 an almost yearly edition has been necessary, is due above all to its efforts to be clear and concise, and to bring forward only reliable and fully proved methods of examination, which are as simple as possible to undertake and to put into practice. Especially intended in the first place for the use of the physician and the veterinary surgeon, it will also help the apothecary and the chemist and those of other callings, as it also includes what is necessary for their requirements.

Through the kindness of the English publishers of this little book, I have been able to look through the translation. I can only express my full approval of the translation, and will only say that the additions in the English edition made to bring in special methods customary in England seem to me to add more value to its contents.

I hope with all my heart that this little book may be of use to the English student and friend of bacteriology, just as it has been able to win for itself a circle of friends in Germany, and that it may assist in spreading and deepening the knowledge of bacteriology for the benefit of the various callings in need of it, and so *pro bono publico.*

BERLIN,
August, 1907.

TRANSLATOR'S PREFACE

In rendering Professor Abel's Bacteriological Pocket Book available for English readers, the opportunity has been taken of including some of the more important methods introduced in the course of recent development of bacteriology in this country.

Dr. Houston has kindly taken charge of the section dealing with the bacteriological examination of water, milk, sewage, etc., and Dr. [illegible] has been so good as to revise the section on methods of removing material from the body for bacteriological examination. The bacteriological examination of dust and air has been dealt with in somewhat more detail than in the original, and a section has been added on methods of examining the blood in relation to immunity. Minor additions inserted elsewhere are enclosed in brackets.

The translator desires to record his thanks to Col. Leishman for kindly supplying the description of his methods of estimating phagocytosis, and of determining the presence of stimulins; and he also wishes to acknowledge his indebtedness to Dr. Louis Elkind for assistance in the work of translation.

London,
August, 1907.

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REFERENCES

A. I. P.	Annales de l'Institut Pasteur.
B. Klin. W.	Berliner Klinische Wochenschrift.
B. M. J.	British Medical Journal.
Centr. f. Bakt.	Centralblatt für Bakteriologie (Or. = Abt. I, Original-Band).
D. M. W.	Deutsche Medizinische Wochenschrift.
Hyg. Rdsch.	Hygienische Rundschau.
L. C. C.	Report to the London County Council.
L. G. B.	Report of the Medical Officer, Local Government Board.
Phil. Trans.	Philosophical Transactions of the Royal Society.
P. R. S.	Proceedings of the Royal Society.
R. S. C.	Report of the Royal Commission on Sewage Disposal.
Zsch. f. Hyg.	Zeitschrift für Hygiene.

Further references in the text are given with sufficient fullness to directly identify their origin.

I

The Microscope

IN bacteriological work dry lenses are used for examining microscopical preparations and colonies of bacteria. For observation of the bacteria themselves an immersion lens is necessary.

When examining a preparation with the *immersion lens* a drop of the immersion fluid is first transferred to the clean (for method of cleansing see p. 7) surface of the cover-glass (usually squares 18 mm. long and 0·16 mm. thick) by means of a glass rod. As a rule the immersion fluid employed is cedar oil (thickened cedar oil, not the thinner sort used for clearing sections). The tube of the microscope is lowered until the objective just touches the drop of immersion fluid. At this point one looks through the microscope and continues to lower the tube by means of the coarse adjustment until an indistinct picture of the object comes into view. Focussing is then completed by means of the fine adjustment.

Bacteria are examined (*a*) in unstained and (*b*) stained preparations.

(*a*) *Unstained preparations* serve for the purpose of studying the characteristics of living micro-organisms, and are made in the form of *hanging drop preparations* in the following way. On the centre of a clean cover-glass which is placed either on laboratory bench or held with a Cornet's forceps, one puts by means of a platinum loop which has been thoroughly sterilized in the flame a drop either of physiological salt solution (0·7%–0·8%) or of broth, or of peptone water (see pp. 13, 22). By means of a sterilized platinum needle a trace (not too much) of the material which contains bacteria is next transferred to this drop on the cover-glass. In cases where the fluid to be examined is not too rich in bacteria, a drop of it may be placed on the cover-glass without dilution. A hanging drop slide is now taken, and the margin of its cavity covered with vaseline. The cover-glass is then placed upon this cavity of the slide in such fashion that the drop lies freely in the hollow. The cover-glass must be firmly pressed down all round on the vaseline so that the drop hangs in a completely closed space; for if this is not the case evaporation occurs, and not only does the drop tend to dry up, but currents are apt to develop in it which drag along the bacteria and thus produce movements which may be mistaken for true motility.

Examination by the hanging drop method has the advantage over the other method of examining fluid directly, e. g. by spreading the material between a cover-glass and an ordinary slide, in that evaporation is more readily prevented, and the material is less liable to escape and infect instruments, &c.

When examining a hanging drop preparation microscopically, in the first place a low power is used in conjunction with a narrow diaphragm, and the preparation is placed on the stage of the microscope n such fashion that the edge of the drop passes directly across the centre of the field of vision. If the low power is replaced by an immersion lens, and the lenses are correctly centred, the edge of the drop will then again pass across the centre of the field; that is to say, it will be readily perceptible. When replacing the low power objective with the immersion lens, therefore, one places a drop of cedar oil on the cover-glass without moving the preparation, widens the diaphragm and lowers the tube until the lens touches the oil, then looks through the microscope and lowers the tube further, using the greatest care, and proceeds slowly downwards with the fine adjustment until the margin of the drop comes into view. It is advisable to move the slide very gently from side to side with the hand, for in this way one is able to perceive at once if the lens has been lowered too far, and by pressing upon the cover-glass threatens to break it. Further, the margin of the drop which in the first instance appears as a shadow like any other object is more easily seen in a state of movement than of rest. Outside the edge of the drop one may observe a fine network due to precipitates resulting from evaporation on the under surface of the cover-glass round the drop.

Experienced observers sometimes examine the drop by an immersion lens straight away; beginners, however, may add to it a trace of very dilute fuchsin solution for the purpose of enabling them to find the drop more easily. If in small quantity, the dye does not interfere with the bacteria. In any case, the edge of the drop is looked for because its outline is more easily perceived than the rest of the drop. In consequence of the more active gas exchange between air and fluid which prevails at the edge of the drop, motile bacteria especially tend to collect round the margin. Distilled water should not be used for hanging drop preparations because it tends to reduce motility. Cultures which have just been taken from the incubator at 37° C. should be examined in a fluid previously warmed, so that the bacteria do not appear non-motile in consequence of rigidity due to cold.

Try to avoid mistaking molecular or Brownian movement, i. e. vibration from side to side, for true motility or change of position. The drop should be as flat as possible in order that it may be accessible in all its parts to the immersion lens, for if the drop is too large, the deeper parts lie external to the focal range of the lens.

If the cover-glass is sterilized in the flame and a droplet of transparent nutrient medium, either liquid or rendered fluid, is placed upon it, a culture preparation is obtained in which the growth of bacteria can be directly followed with the eye. The warm stage is of use in this connexion.

As soon as the examination of a hanging drop preparation is finished, the cover-glass is raised from the glass slide in such manner that the drop does not touch the slide, and the cover-glass with its attached drop is plunged into strong sulphuric acid for the purpose of destroying the bacteria. The glass slide may then be used for another specimen. At the end of the examination the vaseline is removed from the lide by means of blotting-paper, and the slide subequently cleaned with a rag steeped in benzene.

As regards the preparation and staining of film-preparations and sections see pp. 51, 63.

Illumination. In examining the hanging drop, it is well to employ the concave mirror, and either a narrow stop or a contracted iris diaphragm, in order that a contour or refraction image may be obtained. The more powerful the objective, the wider should the diaphragm be opened—it should be about half open if the immersion lens is used. The Abbe condenser is put out of action by application of the diaphragm, so that it is not necessary to remove it. When examining stained preparations, the plane mirror is used without the diaphragm and an absorption or colour image is obtained: in the last case the *Abbe condenser* comes into action, and the latter should be adjusted in such a way that the light refracted by it is thrown directly upon the spot where the specimen to be examined lies. First the specimen is placed in position, under a low power. Then the plane mirror is turned towards an object which lies at some distance—a tree or house for instance—and the condenser is raised or lowered without further moving the tube until one perceives in and upon the image of the specimen a distinct figure of the reflected object. The condenser is left in this position, and now the mirror is turned from the tree or house to any distant source of light which does not yield a reflection of its own, e. g. white sky. The adjustment of the Abbe condenser in the particular way described, however, is not always necessary. In case of a distant l
source it is as a rule sufficient to screw this app
up as high as possible ; but when the source of

is near at hand the condenser ought to be lowered if one wishes to obtain a good effect. If the light is bad, the condenser should be minutely adjusted.

Glaring sky light is relieved by interposing a plane of blue glass in a slot below the stage of the microscope, or by means of blinds to the windows.

As regards artificial light, incandescent light is especially suitable. An oil lamp may also be used provided that its light is made to pass through a blue glass disk in the diaphragm of the microscope, or through a shoemaker's ball, or through a bulb filled with copper and ammonium sulphate. The last solution is made up by adding ammonia to an intensely blue solution of copper sulphate until the light which passes through appears purely white in the microscope. Electric light requires to be relieved by interposing a blue or ground glass screen in the microscope, or by application of a 'frosted pear'. In case of the source of light being near at hand and its reflection interfering with the examination of specimens, one should use the concave mirror.

Directions for cleaning the Microscope.

When removing an immersion specimen from beneath the microscope, one first raises the tube so that the lens is not injured in the process of removing the preparation. On completion of the microscopical examination, the immersion fluid should be removed from the lens by means of fine blotting-paper (Joseph paper); then the lens should be cleansed with a piece of clean wash-leather. If the lens is covered with resinous oil, Canada balsam, or similar material, it may be cleaned with a small piece of leather, or fine blotting-paper wetted either with xylol, benzene, or similar solvent. Care, however, should be taken, for such solvents are apt to dissolve the material with which the lens is cemented.

After use, one protects the microscope against light and dust by placing it either under a dark glass bell jar, or in its box or case. If the objective is not removed, a little piece of fine clean blotting-paper should be placed between the Abbe condenser and the objective to prevent their coming in contact.

For preservation of preparations see pp. 53, 54, 188.

Directions for cleaning Cover-glasses.

1. New cover-glasses are cleaned with alcohol and ether equal parts, or with xylol, or benzene ; and a fine linen cloth or thin blotting-paper are used for this purpose. A drop of water must distribute evenly over the surface of the cleaned cover-glasses ; and if they fail to pass this test, the cleansing process must be renewed, or the cover-glasses treated in the way directed for old ones. Cover-glasses that have stuck together may be heated on a tin plate over the flame.

2. *Old cover-glasses.* Method (*a*) After use, cover-glasses are immersed in a glass jar containing raw sulphuric acid. For the purpose of cleaning, they are poured with the acid into a white porcelain bowl, and after the acid has been poured off, they are thoroughly washed in running water and then boiled in strong caustic soda or potash. On removal from the boiling

alkali they are again washed in water, and subsequently treated with equal parts alcohol and ether, or with xylol or benzene, and cleansed with a piece of linen or thin blotting-paper. The test of cleanliness which all these cover-glasses must pass is that a drop of water placed upon their surface must distribute evenly, and if they fail to pass this test, the process of boiling with caustic potash must be repeated. Method (*b*) According to Kettnow cover-glasses may be cleaned as follows:—(1) They are boiled for ten minutes, stirring the while, in the following solution:—potassium bichromate 200 grams, hot water 2 litres, strong sulphuric acid 200 c.c. (2) The fluid is poured off and the cover-glasses are rinsed for five minutes in a dilute solution of caustic soda. Both processes are now repeated, the first being continued for only five minutes. The cover-glasses are first washed in water, then in alcohol, and finally cleansed by hand.

II

Sterilization and Disinfection

ALL utensils and nutrient media used for cultivation of micro-organisms must be free of germs: otherwise such germs may develop as contaminations.

The following general rules should be observed:—

1. Minor instruments such as platinum needles, knives, scissors, forceps, glass rods, may be sterilized in the flame. Platinum instruments are heated to

redness, but the other instruments need only be heated in such wise that all parts destined to come in contact with the material which contains bacteria are licked over by the flame for at least two seconds. Instruments of metal tend to suffer through heating and to become blunted; the better ones therefore may be sterilized according to the rules laid down in 2 and 3. Only platinum-iridium instruments preserve their edge despite frequent heating: these are useful for subculturing hard or tough cultures (actinomyces or moulds) and also for obtaining blood. Glass plates, smaller glass bowls, and pipettes may also be sterilized if necessary in the flame. Test-tubes, again, may be sterilized in the same way, and should be heated until their protecting plugs of wool are browned. Parts of syringes, if they are not composed of materials liable to be injured by the flame, may also be sterilized in the same fashion.

2. All other major instruments which can stand dry heat, viz.:—glass utensils, metal instruments which are not soldered, wadding, blotting-paper, &c., are sterilized in the hot air sterilizer by being kept for half an hour at 150° C. and over. Wadding should not be heated above 180° C., since it becomes brown and brittle above that temperature. The same applies to blotting-paper, which also becomes brown. Glass and metal articles may be exposed to 200° C. and higher.

3. Materials that do not stand dry heat, but can be boiled without undergoing any change, are sterilized by being heated in steam. To this category belong all the nutrient media (excepting those which contain

pathogenic germs by boiling them for 1-2 hours in a pot of water, or by placing them in the steamer for this time. Hands infected with bacteria should be thoroughly cleansed as follows:—Rinse them in 1:1,000 solution of sublimate and salt, or in Cresol soap solution 1:1,000, next wash and scrub them in soap and water, brush them in alcohol, and then in fresh disinfectant solution and wash again. N.B. The first water used for washing, and the brush, may, under certain circumstances, contain living germs, and should therefore be disinfected if possible. For mode of disinfecting the bodies of infected animals, see p. 188.

III

Nutrient Media

THE basis of media most generally used for the cultivation of bacteria is:—

Meat Extract, or Fleischwasser.

1. 500 grams of finely-chopped beef or horseflesh, as free as possible from fat, are warmed in the pot with a litre of ordinary water at 50° C. for half an hour, and then boiled from ½-¾ of an hour.

2. The fluid is filtered or strained off from the meat, enough water is added to make it up to one litre, and it is then poured into a flask the mouth of which is plugged with wool. From this meat extract nutrient broth, nutrient gelatine, and nutrient agar are made. If it is to be preserved, the meat extract must be

heated on three consecutive days for half an hour at a time in the steamer; or it may be sterilized at once by exposing it in the autoclave for fifteen minutes to 120° C.

Broth.

1. To meat extract prepared as above are added peptone (Witte-Rostock or Chapoteaut Paris) 1% or even up to 5%, and ½% of common salt. For some bacteria the addition of from ¼–1% of grape sugar is advisable, in which case the sugar should be added at the end of the process, because, when boiled for some time, sugar is converted into caramel, which produces brown discoloration of the medium. When the broth has been prepared, the desired amount of sugar should be added from a sterilized 10% solution of it.

2. Heat in the steamer until dissolved.

3. Neutralize either with saturated, with 10%, or with normal solution of sodium carbonate, or with disodium phosphate, or with 25% NaOH. The correct reaction is reached at the point when blue litmus-paper is no longer reddened when a drop of the medium is placed upon it. Red litmus-paper will then be rendered blue. For purpose of comparison, observe the tint produced by a drop of water from the tap. If too much alkalinity has been produced, this may be reduced by adding lactic acid, phosphoric acid, or HCL drop by drop. A small excess of alkali (Na_2CO_3, not NaOH) does, as a rule, no harm (see gelatine, pp. 15, 16).

For neutralization with phenolphthalein as indicator see p. 19.

4. Heat in steamer for $\frac{1}{4}$-$\frac{1}{2}$ hour.

5. Filter. Re-test reaction of the medium, and if necessary correct it. Again steam and filter. Should the filtrate on cooling not be clear, the process of filtration must be repeated, or clear as directed for gelatine (see below).

6. Sterilize in a flask, or else place in tubes and steam on three consecutive days for $\frac{1}{4}$-$\frac{1}{2}$ hour, or autoclave it as directed on p. 10.

Gelatine.

1. To meat extract the same addition is made as in preparation of nutrient broth, together with 10%, or in summer 15%, of the finest white gelatine.

2-5 are carried out in the same fashion as in the case of nutrient broth. If, despite of a correct reaction and thick filter, one does not obtain a fairly or completely clear filtrate, then the white of an egg or 10-20 c.c. of meat juice expressed in the cold from raw meat may be added to the gelatine after cooling it down to about 50° C. Shake well and filter again after careful boiling.

6. Carry out as in the case of broth.

The gelatine remains firm at temperatures between 20-27° C. (see below): at slightly higher temperatures it melts slowly, but at 35° C. it melts rapidly; when in a melted condition the gelatine solidifies again if cooled below 20° C. Gelatine must not be boiled too frequently, or too long, and care is especially necessary when exposing it to a temperature above 100° C. in the autoclave; for though it stands being heated once to 110° C. for fifteen minutes, its capacity for solidifying is apt to be impaired by exposure to such temperatures. The melting-point of gelatine, in fact, remains the higher the less it is heated. The melting-point of recently prepared gelatine is always somewhat lower than that of gelatine that has been left in a solidified condition for twenty-four hours or longer.

Nutrient gelatine with a specially high melting-point prepared according to Forster. In a small vessel containing 1 litre of sterile broth and warmed to 60° C., dissolve 100–150 grams of gelatine. Add KOH to reduce the acidity till it is faint, then make slightly alkaline with Na_2CO_3. Now add the white of an egg. Stand the vessel in a large boiling-pot filled with boiling water and stir the gelatine well. In three minutes it becomes heated up to 98–99° C. Now close the cover of the large boiling-pot, and boil it together with the vessel containing the gelatine for about fifteen minutes. Filter through a warm-water funnel at a temperature of 60° C. (the filter, funnel, and collecting flask having been previously sterilized). Collect the whole of the gelatine in one flask. Transfer it aseptically into sterile test-tubes and heat for twenty minutes in the steamer, then cool by immersion in cold water. The gelatine is now ready for use, and sterile; its melting-point lies after several days between 29–30° C.: but at first is somewhat lower. The principal point of this method is the brief duration of the boiling of the gelatine.

For certain purposes it is well to make the gelatine somewhat alkaline. An addition of 10–15 c.c. of normal soda solution per litre of gelatine over the neutral point scarcely ever does any harm. Certain kinds of bacteria require a still higher alkalinity (see later). The following soda solutions are of equivalent value :—10 c.c. of normal solution of soda, 5·3 c.c. of 10 % solution of water-free soda, or 14·3 c.c. of 10 % solution of crystallized soda. A 10 % solution of crystallized soda contains 3·7 % Na_2CO_3. Normal soda solution contains 5·3 % Na_2CO_3.

[The following is a convenient and quick way of making gelatine. *Composition of the Medium :* Gelatine (yellow gold label) 100 grams (in summer 120), Peptone (Witte) 10 grams, Sodium Chloride 5 grams, Meat Extract to 1 litre. *Directions :*—Put 500 c.c. of the meat extract into a white enamelled saucepan, heat to about 70° C., add peptone, and stir with a small wooden spoon. When the peptone has dissolved, add the gelatine, and stir again till dissolved. Now add remainder of the broth, thoroughly mix, make alkaline with Liq. potassi (till blue litmus-paper is rendered slightly more blue), add the beaten white of one egg, put in a flask and heat in the steamer for 30 minutes. Filter through two thicknesses of Swedish or Rhenish filter-paper moistened with sterile distilled water (placing the funnel in warm filter jacket) into a sterile flask, put into tubes, and sterilize in steamer for 30 minutes on two successive days.]

Agar.

1. To meat extract the same addition is made as in the preparation of nutrient broth, and also 1½–2 % of finely chopped or powdered agar. It is well to add

the agar to the meat extract a couple of hours before the other ingredients, for in this way the agar becomes softened, and dissolves more easily.

2 - 4 are carried out as in the case of nutrient broth. Now test the reaction of the medium and correct it if necessary.

5. The turbid flocculent medium must next be filtered. This is not easily effected through filter-paper, even with the aid of a warm funnel, or in the steamer. Therefore it is better to filter through cotton-wool, as follows: – A fourfold layer of cotton-wool (of the kind used for bandaging) is laid across a funnel in such fashion that the wool projects over the edge. The funnel thus prepared is placed in the steamer for an hour and the hot agar poured on to it. Another method is to omit filtering, and to allow the fluid agar to stand in the steamer after turning out the flame, in which case the turbidity mostly deposits, and the clear supernatant fluid is drained off. Narrow and long cylinders are well adapted for this purpose; for one can allow the agar to solidify therein, and then push it out and cut away the opaque lower portion.

6. As for broth.

Repeated boiling does not injure the capacity of agar for solidifying. Agar melts between 90–100° C., and remains fluid down to 40° C., but if the temperature falls below this point it solidifies very quickly—almost suddenly. When agar solidifies it expresses a little fluid—so-called condensation fluid.

[The following method of preparing ordinar nutrient agar by softening it first with dilute ac acid, has been in use in Dr. Klein's laboratory

(0·5 grm. phenolphthalein in 100 c.c. of 50 % alcohol solution) and titrate with $\frac{N}{20}$ NaOH or HCL until the fluid develops a brilliant red colour. Now add to the rest of the medium according to the result of this titration experiment normal NaOH or normal HCL until the reaction is neutral. Thereupon titrate again a sample of the medium (5 c.c.) as above and correct the reaction of the rest if necessary. Heat to boiling and test again. If the medium is now neutral or slightly alkaline to phenolphthalein it is strongly alkaline to litmus, since the peptone and diphosphate present in the medium are neutral or alkaline to litmus but acid or neutral to phenolphthalein. As media are most suitable for bacterial growth when neutral or slightly alkaline to litmus, media neutral to phenolphthalein must receive an addition of acid. Add 1·5 % (up to 2·5 %, note how much) normal HCL, boil, filter, and sterilize.

4. The addition of 2–8 % of glycerin puriss before sterilization increases for some purposes the value of a medium. Glycerin agar is the usual form of glycerinated medium employed.

The process of filling tubes with media and sterilizing them.

New test-tubes (those in general use are 160 mm. long by 16 mm. in diameter) should be boiled before use in 1–2 % HCl, for the reason that when heated they are apt to give off alkali, which renders the medium turbid and unfit for use. It is advisable to purchase tubes of Jena glass (Schott), which give off but little alkali. Old test-tubes are boiled in water or placed in the steamer. The tubes are cleaned by careful brushing, and are then allowed to dry, after which each is provided with a plug of wool about 3 cm. long, which is gently rolled in and allowed to protrude a little over the upper margin. They are then sterilized in the hot-air sterilizer as directed on p. 9. This sterilization, however, may be omitted in case of tubes that will be subsequently sterilized after being filled with nutrient media.

When pouring medium into tubes, the lower end of a funnel should be provided with a piece of india-rubber tubing fitted to a small glass tube. By means of this appliance (which is sterilized in the steamer with the medium, and provided with a clip at the rubber portion) one can readily fill the tubes without smearing their upper portion and thus causing the wool plug to stick to the glass. Each tube should contain about 5 c.c. of medium.

For filling tubes with definite quantities of medium either a specially constructed filling funnel (Treskow's) is used, or a graduated burette is fitted with a tube junction and a λ or ⊥-shaped nozzle, through one limb of which the fluid medium is allowed to flow up to a given mark. The flow is then shut off; and from the other limb, which is provided with a rubber tube and pressure clip, the required quantity is filled into the tube. Before sterilizing tubes filled accurately i this way, one should as a precaution mark the upp limit of their contents with a grease pencil and obser whether the quantity decreases during sterilization.

blood-serum is needed, it is best obtained in this fashion.

For most purposes one can sacrifice the transparency of the solidified serum (which is on the whole somewhat feeble) and sterilize it after solidification in sloped tubes by heating to 95–98° C. for three successive days for half an hour in the serum inspissator; or one can heat it in the steamer to 100° C. straight away, but in that case the surface of the medium often becomes uneven from the formation of bubbles. However prepared, as a precaution one keeps the tubes before use for twenty-four hours at 37° C. and eliminates those which show contamination (turbidity of condensation fluid, &c.).

Instead of tubes, one may allow the serum to solidify in Petri dishes (p. 97), and sterilize as above, but in this case the surface of the medium dries up quickly, and extraneous organisms tend to find access to it.

Ascites or hydrocele fluid obtained by aseptic puncture can be used in place of serum; the reaction, however, is sometimes very alkaline, and should always be tested.

Blood Serum with Broth (Loeffler's Serum).

The nutrient property of the blood serum is increased by adding to 3 or 4 parts of serum one part of slightly alkaline broth containing peptone 1%, salt 0·5%, and glucose 1%. This addition is made before the tubes are filled. The capacity of the serum for solidifying is not reduced by the addition of the broth, but rather higher temperatures (90–95° C.) must be employed for obtaining good solidification.

Human Blood Serum.

See under Gonococci (p. 148).

Serum-Agar.

Sterile fluid serum warmed to between 40 and 50° C. is mixed with agar (2–3%) which has been melted and then cooled down to this temperature. The serum is mixed with the agar either in equal parts, or in the proportion of 1 of serum to 2 of agar. The medium, which solidifies on cooling, is either inoculated while still fluid and then poured into plates, or else it is first allowed to set in plates and in sloped tubes, and surface cultures are then made. Ascites or hydrocele agar is prepared in a similar manner (see also p. 147).

Eggs.

The shell of the egg is carefully washed and brushed in soap and water; it is then washed in 5% sublimate and in sterile water, and dried with sterile wool. For the purpose of inoculation a small opening is made in the apex of the egg, and when the inoculation has been made through this, the opening is closed with sealing-wax, or sterile paper and collodion. The medium is an unsatisfactory one, because it is frequently not sterile.

Solidified white of egg may be prepared either with or without the yolk. In either case the shell is sterilized. When preparing the white separately, the

Bread.

Very dry white bread is powdered. Sufficient bread crumbs to cover its bottom are placed in an Erlenmeyer flask, and enough water added to produce a thick pulp. The bread medium is then sterilized either in the steamer or in the autoclave. It has an acid reaction, and is a good medium for moulds.

Milk.

Fresh milk, preferably with the cream removed, e.g. skim milk, giving an amphoteric reaction to litmus-paper, is poured into test-tubes and heated in the steamer on three successive days for ½–1 hour. (At a temperature of 110° C. and over, the medium undergoes brown discoloration.) To prove its sterility, milk should be kept for at least three days at 37° C. before inoculation.

Albumen-free Media according to Uschinsky and C. Frankel.

NaCL 5·0, dipotassium or sodium phosphate 2·0, asparagin or aspartate of soda 4·0, ammonium lactate 6·0, dissolved in ordinary water 1,000 c.c. Neutralize and make slightly alkaline with NaOH. Sterilize as in the case of broth.

The Preservation of Media.

General Rules. Always label media with the date of manufacture, composition, and reaction.

Nutrient media frequently deteriorate on keeping, especially from evaporation, and from the penetration of moulds, which are apt to gain access despite a closely-fitting plug. Both these contingencies, however, can be avoided by flaming the margin and protruding plug of the tubes or flasks containing the medium directly after they have been steamed, and by then covering them with a closely-fitting cap of sterilized rubber, tinfoil, or metal. Another way of preserving media is to place the drawer containing them in a tin box (biscuit tin) with closely-fitting top, in which at the same time a large piece of blotting-paper is placed which has been saturated with oil of cloves.

Dried-up media may be renovated by adding a quantity of water equal to that lost by evaporation, and by then resterilizing them after thorough admixture.

Media beginning to get mouldy can also be saved by resterilization.

For special culture media see the following pages:—Drigalski and Conradi's Medium, p. 117, MacConkey's Bile-salt Media, p. 200, Nasgar, p. 147.

IV

Culture Methods

Plate Cultures.

THESE serve for the purpose of isolating from a mixture the different kinds of bacteria which may be present. The mixed bacteria are distributed in a solid medium which has been melted; and thus a held apart when the medium solidifies. Provided that the medium is favourable to them, such separated bacteria then develop into isolated colonies.

1. *Gelatine Plates.* Three tubes of gelatine are liquefied in a water bath at 30–35° C. One of these tubes is held in the left hand between the thumb and the palm which is turned upwards in an oblique position, the opening of the tube pointing to the right. The plug of wood is now removed from the tube and held between two of the finger-tips of the left hand in such a way that the part of the plug which is to be again introduced into the tube points downwards, and is not touched by the fingers. A tube should always be held in this way when it is being inoculated. With a sterilized platinum loop one then takes a trace of the bacteria-containing material and distributes it in the fluid gelatine contained in the upper portion of the tube. The loop is sterilized in the flame again and laid aside, and the plug of wool replaced in the tube. The inoculated material is then distributed in the gelatine by gently moving and rotating the tube. Shaking should be avoided, for thereby bubbles are liable to be developed which become fixed in the gelatine and thus simulate colonies. The fluid gelatine should not be allowed to touch the wool plugs.

Now one grasps the tube in the same way as before, and also the second tube, and removes the plugs from both. With a sterile platinum loop three drops of tube 1 are then transferred to tube 2. The loop is resterilized, the plugs replaced, and tube 2 gently rotated to ensure distribution of the material. Tube 1 loopfuls

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Abel, Rudolph.
Abels Laboratory handbook of bacteriology. tr. from
the 10th German Ed. by M. H. Gordon. London, Oxford.
224 p. 1907.

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fully with a grease pencil, or better with a label, not omitting to record the date.

2. *Agar plates* are made in just the same way as gelatine plates, the agar being melted and then cooled to 40° C. Work quickly because the agar sets rapidly. It is well to warm the plates slightly before pouring in the medium. Roll-tubes of agar turn out badly. Agar plates are inverted before they are placed in the incubator because otherwise the condensation fluid which is expressed from the agar is apt to make all the colonies on the surface of this medium confluent.

For preparation of serum-agar plates, see p. 25.

Advantages of gelatine. Colonies mostly grow in characteristic forms.

Disadvantages. It remains firm only to 30° C. at the outside; and made in the usual way it only remains firm up to 22–24° C., especially in plates. It is therefore not solid at the body temperature. The rapid liquefaction (peptonization) of gelatine produced by certain bacteria also interferes with the value of plates of this medium for isolating bacteria.

Advantages of agar. It can be used as a solid medium for the culture of bacteria at the body temperature. It is not liquefied, so that agar plates keep longer than gelatine.

Disadvantage of agar. The bacterial colonies are not as a rule characteristic.

An Economical Method of diluting Culture-material.

If one wishes to obtain dilutions with small consumption of gelatine or agar, one places a number of drops of broth or gelatine on a sterile plate and inoculates the first drop with the material to be examined, from this the second drop, from that the third, and so on. From given drops, one or more gelatine or agar tubes can then be inoculated and poured out as plate cultures.

The Examination of Plates.

Colonies developing in plate cultures are examined with low objective, narrow diaphragm, and concave mirror. In the case of Petri dishes one raises the cover; or, if one does not wish to subculture, the plate may be examined microscopically in inverted position.

Isolated colonies may be subcultured by the process known as *Fishing*. The selected colony is placed in the field of a low power microscope, and with a fine sterile platinum needle, the end of which may with advantage be bent, one touches lightly this colony, controlling the process by looking through the microscope. After the colony has been touched, the needle is removed as carefully as when brought to the colony. In order to avoid touching any other part of the plate than the selected colony, the little finger of the hand that guides the needle may be rested on some solid support. The material removed from the colony by the needle is used either for preparations or for pure cultures.

A more comprehensive investigation of the growth on a plate may be effected by means of *Impression-preparations*, which are made in the following way. A cover-glass is sterilized in the flame, allowed cool, pressed firmly down on the plate and then raised with the forceps. The preparation thus obtained is stained according to the directions given later (p. 51). Impression-preparations afford general information concerning the kinds of bacteria present, and are especially useful for the study of the smallest colonies. Subcultures must not be made from a part of the plate where an impression-preparation has been made.

For *Enumeration of Micro-organisms* see pp. 188, 192.

The Preparation and Subculture of Pure Cultures.

Preparation from plate cultures. One fishes some material from an isolated colony and inoculates therewith tubes of sterile medium. In fluid media one cleanses the needle by moving it from side to side; in the case of solid media one either moves the needle over the whole of the surface, or one draws it along the middle in a straight line—*Streak Culture.* Avoid scratching the surface of the medium. *Stab Cultures* are made by puncturing transparent medium which has solidified in a tube kept in an upright position. If the growth on the stab is to be examined under the low power of the microscope, the stab is made near the wall of the tube; otherwise it is made in the centre.

Subcultures are made by transferring small quantities of the growth with the sterilized platinum needle or loop into fresh tubes of culture media. When making subcultures, the tubes are held in the manner previously described, gelatine tubes being held with the open end facing downwards.

For the purpose of keeping bacteria in culture, transference to fresh media, or, as the process is termed, subculture, is as a rule sufficient every 4-6 weeks; but some of the pathogenic bacteria (influenza, gonococcus, pneumococcus, and meningococcus) require more frequent transference. Bacteria, however, will retain their vitality for months when one saturates small silk threads (No. 4 size) with cultures of them, or better, with blood or other albuminous fluid in which they are contained. These threads are dried in sterile capsules in a desiccator over $CaCl_2$, and are then kept in small test-tubes which are placed inside larger tubes having $CaCl_2$ at the foot and closed with a plug of wool and rubber cap.

On the employment of Surface Plate cultures for the cultivation of fractions of the material under examination.

Instead of distributing the bacteria-containing material in melted nutrient media, and then pouring this out into plates and allowing it to set, thus fixing the individual bacteria apart, one can effect the isolation of such bacteria by spreading out the material on the surface of media which has already set either in plates or in sloped tubes.

This is effected by using a loop, or a sterile plug of wool, or a heated platinum wire (the latter tends to scratch the surface of the medium), and by this means distributing the material over the whole of the surface of the medium. Provided that the original material is not too rich in bacteria, in the case of plates, where the surface of the medium exposed is comparatively large, it is usually easy to obtain isolated colonies by sowing a single plate. In sowing such a plate a drop is placed on the medium with a loop, and this drop is then thoroughly distributed with a wire bent parallel to the surface of the medium: glass rods bent to a right angle are also suitable for distributing material over a plate. In the case of sloped tubes, however, it is as a rule necessary to inoculate a series in succession, using the condensation fluid as diluent. Thus in inoculating a series of serum tubes one would add a loopful of the material to the condensation fluid of the first tube, and then distribute a loopful of this condensation fluid over the whole surface of the serum in this tube; then with the same needle, without recharging it, a second and third serum tube are dealt with in the same way in quick succession. The third tube will generally be found to yield a crop of well-separated colonies from which pure cultures can be made. This is the sole method of isolating bacteria in pure culture from media which cannot readily be converted from a solid to a fluid state and vice versa, such as blood serum, and potatoes.

The Animal Body as a means of obtaining Pure Cultures.

Some pathogenic micro-organisms can be isolated from mixtures by means of the animal body. For this purpose it is necessary that no other pathogenic germs which can penetrate to the inner organs of the animal should be present in the mixture. In a successful case, after inoculation, only one kind of micro-organism multiplies in the tissues of the animal, and after the animal has succumbed, this organism is recovered from its inner organs in pure culture. The method is applicable for the isolation of anthrax, mouse septicaemia, tuberculosis, plague, and the pneumococcus. In making cultures from the animal body, it is generally better not to attempt to make pure cultures direct, but first to prepare plates. If one does attempt to make pure cultures direct, stab cultures should not be made, but slope cultures, in which contaminations are more readily perceived if present. Never cultivate in fluid medium, because here impurities are not so easily recognized.

Hanging Drop Cultures.

These are made in broth, gelatine, &c., for the purpose of directly observing the growth of bacteria under the microscope. For method of preparation see p. 1.

The Cultivation of Anaerobes.

To media employed for the purpose of cultivating anaerobic bacteria one adds as a matter of routine 1-2 % grape sugar, and, as well, 0·3-0·5 % sodium formate, or 0·1 % sulphindigotate of soda. The latter stains the medium blue, but owing to a process of reduction, the colour is usually discharged by the growth of bacteria (p. 46).

A good proportion of anaerobic bacteria are spore-which contains a definite proportion of the disinfectant.

The bactericidal power of a disinfectant is most simply tested by exposing either small pieces of glass, or silk threads upon which the bacteria to be tested have been dried, to solutions of different strength, and allowing contact for various periods. Before the material thus treated is sown in nutrient solution, it should be washed in sterile water, or in a solution that binds the disinfectant but has in itself no disinfectant value; or it should be sown in a rich nutrient fluid and thoroughly shaken.

[Before drawing inferences as to the practical value of a disinfectant, its germicidal action in the presence of albumen should be determined.]

11. Pathogenicity.

For examination of this see p. 180.

12. Toxin-formation.

Toxin may be dissolved in the medium (soluble toxin), or it may be confined to the bodies of the bacteria (endotoxin). *Soluble toxin*:—To demonstrate the presence of this, cultures in fluid media are filtered free of bacterial bodies (see p. 11, 4. (*c*)); the filtrate is then tested on animals (see p. 180). *Endotoxin*:—Cultures on solid media are killed by chloroform or toluol vapour. This may be effected by pouring a few drops of chloroform or toluol on to the under side of the plug of wool with which the tube is plugged, by covering the tube with a double rubber cap, and then placing it for one to several hours in the incubator at 37° C. (Take care that the other cultures in the incubator do not suffer.) The killed growth may then without any admixture of medium be inoculated into animals. A control culture should be made to prove that all the germs are killed. (For dose to be inoculated see p. 182.)

[Endotoxin can be liberated from the bodies of bacteria by promoting autolysis (e.g. by NaCl); by dissolving their substance in NaOH; or by grinding them up in a special apparatus (McFadyean and Rowland) at the temperature of liquid air which apparently renders them brittle.]

V

Staining Methods

The Making of Preparations.

1. Cover-glass, Smear, or Film Preparations.

The Film. On the centre of a carefully-cleaned cover-glass (for slide preparations see p. 54) a drop of water is deposited by a platinum loop, and to this drop is added by means of a loop or needle a trace of the material under examination. The drop is then spread out over the surface of the cover-glass to form a uniform thin film. This is only possible when the cover-glass is absolutely free of grease. The cover-glass is then allowed to dry, and this may be accelerated by gently warming it over the flame, with the film side upwards.

Fluids that do not contain too many bacteria—blood, pus—are smeared directly over the cover-glass undiluted. In the case of organs, a small piece is seized with sterile forceps and lightly smeared on a cover-glass. Materials which distribute over the cover-glass with difficulty, such as some kinds of sputum, nodules of tumours, and certain cultures, can be squeezed between two cover-glasses which are freed from one another by holding them in a pair of Cornet's forceps. Take care to avoid smearing the fingers. (For impression-preparations see p. 33.)

Fixation. The cover-glass with the film side upwards is held between the finger and thumb (or if the edge has been smeared, in the forceps) and passed three times through the flame. The advantage of holding the preparation between finger and thumb is to ensure that it is exposed to the right temperature. After being fixed in this way the film is ready for staining. Fixation can also be effected by alcohol; but the process takes longer. In case of blood preparations, it is recommended that they should be fixed by being left in equal parts of alcohol and ether from 2–10 minutes or longer.

Should doubt arise as to which side of the cover-glass the film is on, a hint may be gained by breathing on it; the moisture condenses unevenly on the film side, evenly on the other. Or the cover-glass may be scratched with a needle.

Staining. For purposes of staining it, one takes hold of the cover-glass with a Cornet's forceps—an instrument usually closed, but opening on pressure. With a pipette (which should not be allowed to touch the film) one then drops on to the cover-glass sufficient stain to cover the whole of its upper surface. As a rule, one allows the stain to act for five minutes in the cold, or for 10–60 seconds over the flame (stains, p. 61). Another method of staining is to allow the cover-glass to float film downwards on some stain in a watch-glass; and in cases where prolonged staining is needed the watch-glass may be warmed on the wire gauze of a tripod.

Washing. After having been stained, the cover-glass preparation is washed in tap-water, dried with blotting-paper, mounted in water, and examined microscopically. If, on microscopical examination, parts of the preparation move about and vibrate, it is a sign either of under-fixation or of over-washing. Should the water between slide and cover-glass evaporate, it can be renewed by placing a drop of water at the edge of the cover-glass. Should it be necessary to treat the preparation with other fluids after staining, one either drops these on to the film, or dips the cover-glass into a vessel filled with such fluid.

Mounting in balsam. If desirable, cover-glass preparations can be examined in balsam. In this case, after the preparation has been stained and washed, it is dried either by allowing it to drain on a piece of blotting-paper and then to dry in the air, or by pressing it between two pieces of blotting-paper and then lightly brushing it with a camel's-hair brush. In the last case care must be taken not to injure the film. Having dried the preparation, a drop of viscid Canada balsam (dissolved in xylol) is placed upon the film side of the cover-glass, and the latter placed upon a clean slide. The balsam ought not to run out at the edges of the cover-glass, but should it do so, the exudation may be wiped off after a few days by a piece of blotting-paper steeped in xylol. In the same way immersion oil may be removed from the surface of the cover-glass. When mounted in balsam, bacteria appear smaller than in water, and some peculiarities, e.g. capsules, disappear. It is a good rule therefore always to examine in water first.

Preparations mounted in balsam keep for an indefinite time. They should be preserved in the dark. The stains gradually fade, especially methylene blue. Always label preparations at once.

When it is wished to mount in balsam a cover-glass preparation that has been examined in water, one removes any immersion oil from its surface by means of blotting-paper, runs water round the edge of the cover-glass, and floats it off the slide. The cover-glass is then dried and mounted in the fashion described above.

Slide, i.e. Object-glass Preparations.

These may be made in the same fashion as cover-glass preparations, and are recommended in cases where one has to examine a large quantity of material and has only a single occasion on which to do so. The film is fixed and stained in the same way as in the case of cover-glass preparations. The slide is then dried with blotting-paper, or in the air, immersion oil is dropped on to it, and the film is examined direct without using a cover-glass. Should it be wished to preserve the preparation, the most important part of the film is covered with a drop of balsam and a cover-glass dropped on to it.

An estimate of the size of bacteria is most simply made by comparing them with red blood corpuscles the diameter of which varies from 7-9 μ. In cases where red blood corpuscles are not present in the material, e.g. in preparations from cultures, a drop of blood from the finger may be mixed with it.

2. Sections.

Hardening. Small pieces of tissue— at the outside as big as a phalanx – are hardened for at least three days in absolute alcohol ; the latter being frequently changed. It is well to place a pad of blotting-paper on the bottom of the vessel, so that the pieces of tissue by resting upon it may be kept in the upper layers of the alcohol, which are more free of water than the lower. Pieces of tissue can be preserved for years in this way. In order to fix them better, one can, if necessary, place them in formalin for 12-24 hours before putting them into alcohol. (For a quick method see p. 58.)

For cutting, a microtome is used, and one endeavours to obtain very thin sections. The pieces of tissue, after being hardened, are attached or imbedded; and for this purpose the following methods are recommended; of which 1 and 2 should be used only for firm and parenchymatous tissue, 3 and 4 more especially for cavernous tissue, or small pieces of tissue.

1. *Attachment by glycerine-gelatine.* 10 grams of

gelatine are dissolved in glycerine 40 + aq. 20 by heat. A drop of this is placed on a bit of cork, the piece of tissue is pressed upon it, and, after two minutes, the whole is placed in absolute alcohol. In the course of a few hours the glycerine-gelatine solidifies, and the material can then be cut. The sections are placed in a capsule containing 50% alcohol, and the knife also should be moistened with the latter.

2. *Imbedding in Celloidin.* After being hardened in alcohol the pieces of tissue are placed for 1-8 days in a thin solution of celloidin made up by dissolving the latter in equal parts of alcohol and ether. They are next placed for the same length of time in a more concentrated solution of celloidin, and are then, by means of a spatula, transferred with the attached celloidin to pieces of cork or wood. The imbedded material must not be pressed upon when moving it. When, after some time, the celloidin has dried somewhat in the air, the pieces are placed in 50-60% alcohol (not absolute) in order to allow the celloidin to solidify. Twenty-four hours later sections may be cut. The sections are kept in a capsule filled with 50% alcohol, and the knife is moistened with the same.

3. *Imbedding in paraffin.* The hardened pieces of tissue are placed for from several hours to days (until they become transparent) in xylol, then for the same time in paraffin dissolved in xylol, then in melted paraffin at a temperature of about 50° C. (Paraffin with the required melting-point can be obtained by ixing different samples.) The pieces of tissue are pt in the paraffin oven at 50° C. for 1-2 hours. After ing soaked in this way in the paraffin, the piece

of tissue is taken out with a spatula and placed on a clean glass plate inside a small mould made of glass, metal, or papier-maché, and melted paraffin is then poured round and over it in such a way that the piece of tissue lies in the middle of the mould. The glass plate may be dipped into water to hasten solidification of the paraffin. If they are not to be cut at once, the paraffin blocks are labelled (by pinning on a label). Before cutting, one pares off the paraffin up to the immediate vicinity of the imbedded tissue in order to see better in which direction it is to be cut. The piece of tissue is then fixed on the block of the microtome and cut with the knife, which is dry. The sections are placed in a bowl of water warmed to between 40-45° C., and at once smooth themselves out. They are now removed from the water by inserting clean slides beneath them and lifting them out in such fashion that they lie smooth on the slide. The slide is then stood on end to drain off the water, and the drying is completed by placing it in the incubator at 37° C. The slide with sections attached to it is placed in the paraffin oven until the paraffin melts and begins to run off. The paraffin is then removed by xylol, and the latter removed in turn by absolute alcohol. The section sticks firmly to the slide.

4. *Freezing in oil of Aniseed.* The pieces of tissue are freed as much as possible from alcohol by drying them between blotting-paper, and they are then placed for at least twenty-four hours in aniseed oil. The pieces of tissue are immersed in aniseed oil in tightly-fitting glass bottles in the incubator at 37° C.

3. Cedar oil or xylol, &c.

(*c*) *With gentian violet.*

1. Stain in watery solution of gentian violet for $\frac{1}{4}$–$\frac{1}{2}$ hour.

2. Wash first in 50 % alcohol, then in absolute alcohol until the section has a bright violet colour.

3. Clear in cedar oil.

(*d*) *Kühne's carbol-methylene blue stain.*

1. Stain in carbol-methylene blue for half an hour.

2. Wash in water.

3. Decolorize in water containing 2 drops HCL to 100 aq. until a pale blue tint is obtained.

4. Wash in water with 3–4 c.c. of saturated watery solution of carbonate of lithia to 100 aq.

5. 3–5 minutes in pure water.

6. Half a minute in absolute alcohol, which may have a little methylene blue powder added to it.

7. Methylene blue aniline oil. (This is made up by dissolving sufficient of the dye to cover the point of a knife in 10 c.c. of aniline oil, and adding aniline oil until the fluid assumes a deep violet colour.) The preparation is left in this stain for several minutes.

8. Wash in fresh aniline oil.

9. Turpentine, xylol, balsam.

Shortening of the Method according to Pregl.

1. Stain in carbol-methylene blue for $\frac{1}{2}$–1 minute.

2. Wash quickly in water.

3. Decolorize in 50 % alcohol until the section becomes pale blue with a slight tinge of grey.

4. Dehydrate in absolute alcohol.

5. Clear in cedar oil, &c.

(*e*) *Methylene-blue-tannin method of Nicolle.*

1. Stain with either alkaline methylene blue or carbol-methylene blue as under (*a*) and (*d*).

2. Wash in water or in ½–1 % acetic acid for a few seconds.

3. Transfer to 10 % tannin solution (whereby the methylene blue is rendered insoluble) for a few seconds. (1 % tannin, as a matter of fact, will do.)

4. Wash in water, dehydrate in alcohol, clear in oil, &c. This method may be recommended for bacilli readily decolorized, such as typhoid, glanders, &c.

(*f*) *Nicolle's thionin stain.*

1. Stain in thionin solution for ½–1 minute (10 parts of saturated solution of thionin in 50 % alcohol + 100 parts of 1 % carbol water).

2. Wash in water.

3. Decolorize in absolute alcohol, then pass through oil, &c.

This method, like (*e*), is recommended for bacteria that easily part with their stain.

The methods (*a*), (*b*), and (*f*) are to be preferred because of their greater simplicity.

Staining of the Bacteria alone, or Contrast Staining.

I. *The method of Pick-Jacobson.* For film preparations. Stain for 8–10 seconds at the utmost in a mixture of Ziehl's carbol-fuchsin (p. 62) 15 drops, saturated alcoholic methylene blue 8 drops, aq. dest. 20 drops.

The bacteria stain dark blue, nuclei bright blue, the rest of the tissue red. (N.B.—If old, this stain is improved by the addition of a little carbol-fuchsin.)

II. *Eosin and methylene blue.*

For film preparations.

1. Stain for half a minute in a freshly-made mixture of Loeffler's methylene blue (p. 61) 30 parts, and saturated alcoholic solution of eosin 10 parts.

2. Wash in water.

Bacteria and nuclei stain blue, cell protoplasm, &c., red.

III. *May Grünwald's method* (*Zentralbl. f. inn. Med.*, 1902, No. 11).

Several litres of 1 : 1000 watery solution of eosin are mixed with the same quantity of a solution of methylene blue (medicinale, Höchst), of similar strength, and after a few days filtered. The portion that remains on the filter is washed with water until this passes away almost colourless. From this precipitate, when dry, a saturated solution in methyl alcohol is prepared and used for staining. The film preparations are not fixed. They are stained in the cold for a few minutes up to as many hours, and are then washed in water containing a few drops of the solution. The stain can be obtained from Dr. Schwalm, München, Sonnenstr. 10. It is especially used for preparations of blood and pus. Assmann (*M. M. W.* 1906, No. 28) lays his preparations in Petri dishes, pours over them forty drops of the solution for three minutes; adds thereupon 20 c.c. of distilled water + 5 drops of 1 : 1000 K_2CO_3, shakes, and allows the stain to act for five minutes; then, without washing, dries, &c.

IV. *Weigert's stain.* For sections.

1. Stain in watery alcoholic gentian violet for 5–10 minutes.

2. Wash in alcohol for 5–10 seconds.

3. Wash in water for five seconds.

4. Counter-stain in picrocarmine (p. 72) for 1–12 hours.

5. Wash in alcohol.

6. Clear in cedar oil, &c.

Bacteria stain blue, cell nuclei red, rest of tissue red to yellow. This stain is not to be recommended for bacteria that are easily decolorized, e. g. typhoid, glanders, fowl cholera, &c.

V. Gram's Stain.

Since only certain kinds of bacteria are stained by it, Gram's stain is used not only for the demonstration of bacteria, but also for diagnostic purposes.

(*a*) *For film preparations.*

1. Stain with warm aniline water gentian violet, or aniline water methyl violet (p. 62), for at least two minutes. (The best stains are methyl violet, Höchst 6 B and BN.)

1 *a*. Wash in aniline water. (This may be omitted.)

2. Place for thirty seconds to two minutes in iodine and potassium iodide solution. (This solution is made up as follows:—iodine 1 part, potassium iodide 2 parts dissolved in aq. dest. 5 parts. After solution, add aq. dest. 295 parts.) When placed in this solution there develops in the bodies of certain kinds of bacteria (see p. 69) a combination between the stain and iodine that is insoluble in alcohol.

3. Decolorize the preparation in absolute alcohol

until no more colour is given off. Those bacteria that are capable of being stained by Gram are now found to be stained blue-black—all other bacteria and the tissue elements (excepting some of the cell nuclei) are unstained. The preparation can at this point therefore be examined in water, or it may be dried and mounted in balsam.

4. Counter-stain with watery alcoholic solution of safranin or fuchsin for several seconds; or with picrocarmine (p. 72) for 2-10 minutes. Counter-staining with fuchsin has the advantage that any bacteria present in the preparation that may have parted with the violet stain are much better stained with it than with safranin or carmine.

5. Wash in water.

Bacteria blue-black; tissue red.

(*b*) *Sections* (see remarks under (*a*)).

1. Stain in aniline water gentian violet or in aniline water methyl violet for 5-30 minutes.

1 (*a*) Wash in aniline water for thirty seconds. (This may be omitted.)

2. Transfer the preparation to the potassium iodide solution for 1-2 minutes. The sections here become dark brown.

3. Wash in absolute alcohol until the section appears entirely or partly unstained. At this stage the bacteria positive to Gram alone are stained, and are blackish-blue in colour. The preparation may then be either cleared in cedar oil, &c., or it may be counter-stained in order to bring out the tissue and bacteria which have not stained by Gram.

4. Wash in water, and then counter-stain with picro-

carmine (p. 72) or safranin, or with dilute fuchsin, for 5-10 minutes.

5. Wash in 60 % alcohol.
6. Dehydrate in absolute alcohol.
7. Clear in cedar oil, &c.

If desirable, one may carry out 4 and 5 first, and then 1-3 followed by 7.

Gram-positive bacteria blue-black, tissue red (cell nuclei often pale blue to dark blue). Bacteria are frequently not stained equally well in all parts of the preparation.

Modifications of Gram's method.

(*a*) Instead of making up the stains with aniline water, one can use 1-2½ % of carbol water, in which case they keep longer. After staining, the preparations should be washed in carbolized water of corresponding strength. The addition of one-tenth part of a saturated alcoholic solution of methylene blue is also to be recommended for decolorizing.

(*b*) For accelerating decolorization according to Günther, alcohol absolute + 3 % HCl may be used for ten seconds followed by absolute alcohol; and according to Nicolle, alcohol absolute + 20-30 vols. per cent. of acetone. (Nicolle recommends carbol water gentian violet made up with 1 % carbol water, and potassium iodide solution of the following composition :—1 K + 2 Ki + 200 Aq.)

The following micro-organisms stain by Gram's method, i. e. continue to remain blue-black after treatment of the preparation with alcohol:—Anthrax, tetanus, tubercle, leprosy, diphtheria, swine erysipelas, mouse septicaemia, the pyogenic streptococci and staphylococci, Fraenkel's pneumococcus, Micrococcus tetragenus, Streptothrix actinomyces, yeasts, potato bacillus, and others. The stain, however, is only useful for differentiating bacilli such as tetanus, tubercle, or leprosy, when it is certain that there are no other Gram-positive bacilli present as well in the tissue. If the treatment with alcohol is prolonged too far, some of the above Gram-positive micro-organisms become decolorized.

The following micro-organisms do not stain by Gram's method, i. e. part with their stain when the preparation is treated with alcohol:—Typhoid, coli, and similar bacilli. Cholera and similar vibrios, fowl cholera, and rabbit septicaemia. B. malignant oedema, and the bacillus of quarter evil or rauschbrand (the latter two are said to occasionally remain positive to Gram), Friedlander's bacillus, plague, glanders, influenza, spirillum of relapsing fever, gonococcus, and meningococcus.

When examining the reaction of a micro-organism to Gram's stain one always uses a young culture, and as a control puts on a part of the preparation in the case of bacilli a smear from a young Staphylococcus aureus culture, or in the case of cocci a smear from a young anthrax culture. Then, if the stain is applied correctly, the aureus or anthrax are stained blue-black. The treatment with alcohol must not be too short, because in that case all bacteria will be found to be stained. If desirable, the control may be carried further by also putting on part of the preparation a smear from a young culture of a micro-organism negative to Gram's stain, e. g. coli, typhoid, or cholera

VI. *Weigert's stain for sections*—the so-called Fibrin method.

1. Stain in lithium carmine (carmine 2·5–5 dissolved in saturated watery solution of lithium carbonate 100) or in watery alcoholic solution of safranin, for $\frac{1}{4}$–$\frac{1}{2}$ hour. If desirable, a stain composed of Ziehl's carbol-fuchsin 1 + aq. dest. 4 may be used instead.

2. Wash in 50 % salt solution.

3. Stain in aniline water gentian violet for 5–30 minutes—or according to Kühne the following stain is preferable: crystal violet 1, dissolved in absolute alcohol 10; of this 1 + aq. dest. 10 + 2 drops HCL.

4. Wash in 0·6 % salt solution.

5. Transfer the section to a slide and dry it with blotting-paper.

6. Place it in potassium iodide solution for 1–2 minutes.

7. Dry with blotting-paper.

8. Decolorize in aniline oil till the oil becomes no longer stained. The staining of the section is controlled by examining it microscopically.

9. Remove the aniline oil with xylol.

10. Canada balsam.

The bacteria are stained violet blue, fibrin deep blue, tissue red. Picrocarmine is not particularly well suited for the preliminary staining of the tissue, since the aniline oil extracts the picric acid from the tissue.

VII. *The stain of Claudius.* This method yields the same result as Gram's method (the picric acid acts like the potassium iodide solution), gives less precipitate than Gram, and stains the bacilli of malignant oedema and rauschbrand blue-black.

(*a*) *For films.*

1. Stain in watery solution of methyl violet for one minute, then wash in water and dry with blotting-paper.

2. Wash in saturated watery solution of picric acid + aq. dest., equal parts.

3. Wash in water, dry with blotting-paper.

4. Wash in chloroform or oil of cloves till the preparation is decolorized.

5. Dry with blotting-paper, mount in balsam.

(*b*) *For sections* (best carried out on the slide).

1. Stain as above for two minutes.

2. Wash in water, dry with blotting-paper.

3. Picric acid as above for two minutes.

4. Wash in water, carefully dry with blotting-paper.

5. Treat with oil of cloves till the section is decolorized.

6. Xylol, Canada balsam.

Bacteria blue; tissue unstained or yellowish, but if first counter-stained with carmine can be rendered red as in case of Gram.

Picrocarmine for counter-staining tissue in the methods of Gram, Weigert, or Claudius.

(*a*) *Prepared according to Friedlander.*

Dissolve carmine 1 in aq. dest. 50 + ammonia 1. Add saturated watery solution of picric acid until the precipitate that forms no longer dissolves on shaking. The addition of a little ammonia will redissolve the precipitate. To prevent growth of bacteria in the stain a few drops of carbolic are added. Filter before use. The stain will keep.

(*b*) *Prepared according to Weigert.*

Allow carmine 2 parts + ammonia 4 parts to stand for twenty-four hours. Then add 200 c.c. of concentrated watery solution of picric acid. Twenty-four hours later add acetic acid drop by drop till a precipitate forms, then ammonia drop by drop till the solution is clear.

Capsule-staining.

In cover-glass preparations many bacterial capsules take the stain when the preparation is warmed for a more or less long time in Loeffler's or Ziehl's stains (pp. 61, 62) : the capsules in the former case staining blue and in the latter violet. Among special methods for staining capsules are the following :—

(*a*) *Friedlander's method for films.*

1. Dip the cover-glass for 1–3 minutes in 1 % acetic acid.

2. Drain off the acetic acid and quickly dry.

3. Stain for a few seconds in saturated aniline water gentian violet.

4. Wash in water and examine therein. In case of the capsule being stained so deeply as to obscure the body of the bacteria :—

5. Wash in 1 % acetic acid or 50 % alcohol for ten seconds.

6. Wash in water, examine therein, and if the stain is successful, dry, and add balsam.

For sections.

1. Stain for twenty-four hours at 37° C. in concentrated alcoholic gentian violet 50 parts + aq. dest. 100 + glacial acetic acid 10.

2. Differentiate in 1 % acetic acid.

3. Dehydrate in alcohol, oil, &c.

When 2 is not sufficiently carried out, the capsules are stained as intensely as the bacterial bodies and hide them.

(*b*) *Ribbert's method* (*for films only*).

1. Stain the cover-glass for a few seconds in the following solution:—Aq. dest. 100, alcohol abs. 50, glacial acetic acid 12·5 + dahlia as much as dissolves on heating.

2. Wash in water and examine therein, or dry and mount in balsam. The latter renders the capsules less distinct.

(*c*) *Bunge's method for staining flagella* (p. 78).

(*d*) *Nicolle's method for films or sections.*

1. Stain with the following mixture:—Saturated solution of gentian violet in 95 % alcohol 10 parts + 1 % carbolic water 100 parts.

2. Wash in absolute alcohol + $\frac{1}{3}$ its volume of acetone. Then in case of cover-glass preparations wash in absolute alcohol, or in case of sections pass through oil, &c.

(*e*) See for further methods under anthrax bacillus, p. 82; these are also applicable for several other bacteria.

Spore-staining.

(*a*) 1. Stain the cover-glass preparation in aniline-water fuchsin or in carbol-fuchsin, heating strongly.

(Stain for one hour if the preparation is floated on the stain film downwards, or for ten minutes if the stain is dropped on to the surface of the cover-glass, adding fresh stain to replace any lost by evaporation.)

Better penetration of stain into the spores is effected if the preparation is passed thirty or forty times through the flame in the fixing.

2. Wash in alcohol + 3 % HCL (or 1 % H_2SO_4, or 3 % HNO_3) for $\frac{1}{2}$ – 1 minute.

3. Counter-stain in watery alcoholic solution of methylene blue.

4. Wash in water and examine therein, or dry and mount in balsam.

Spores red, bacilli blue.

(*b*) Preferable because more reliable is the method of Möller.

1. After fixing it, place the preparation for from five seconds to ten minutes in 5 % chromic acid. The exact time of exposure for each micro-organism must be found by experiment.

2. Wash in water.

3. Place the preparation in aniline-water fuchsin or in carbol-fuchsin and heat to boiling-point.

4. Decolorize for five seconds in 5 % H_2SO_4.

5. Wash in water.

6. Counter-stain with methylene blue.

Prior to treatment with chromic acid the preparation may be placed for two minutes in chloroform to remove fat-drops, &c., which may simulate spores; the cover-glass is then washed in water.

Instead of treatment with chromic acid, the spore membrane may be macerated in a concentrated solution of iodine and zinc chloride, or in a solution of hydrogen peroxide, though both are less active than chromic acid.

According to Aujeszky (*Ctrbl. f. Bakt.*, Abt. I. Bd. 23, p. 329) the air-dried unfixed film preparation should be placed for 3-4 minutes in hot ½ % HCl, washed in water, dried, fixed, and then stained as in 3–6 above.

Flagella-staining.

Having seen that the organism exhibits definite motility in the hanging drop, a portion of its growth on some solid medium is taken for flagella-staining. Fluid media should not be used ; a young agar culture is especially suitable. The bacteria should lie as separate as possible on the cover-glass, and the film should contain as little of the nutrient material as possible. This may be effected in the following way :—A droplet of water is laid upon each of six cover-glasses, which must have been most carefully cleaned. Some growth from a culture is transferred to the first drop, from this a trace is transferred to the second, from this to the third, and so on. Another way is to add a little bacterial growth to a droplet of water on a slide, and to transfer from this a trace to a larger drop of water to which 1-2 loops of 2 % osmic acid solution have been added. From this drop one prepares cover-glass preparations. A third method is to make a thin suspension of the bacteria in 0·8 % NaCl solution in a test-tube, to transfer droplets from it to cover-glasses, and spread them out without rubbing. The cover-glasses are allowed to dry in the air and then fixed by passage through the flame.

The cover-glasses must always be absolutely clean. (For method of cleaning, see p. 7.)

A. Loeffler's method of Flagella-staining.

1. Place some of the mordant described below upon the cover-glass and warm till steam arises.

2. Carefully remove the mordant by washing in a stream of water. If necessary use blotting-paper.

3. Wash in alcohol until only those parts where the bacteria lie appear stained.

4. Stain in aniline water fuchsin and apply heat. The fuchsin solution is prepared according to the directions given on p. 62, and to it is added 1% of a 1% or stronger solution of caustic soda until a precipitate begins to form—i. e. the solution begins to become turbid.

5. Wash in water.

The mordant consists of the following mixture:—10 c.c. of a 20% solution of tannin, 5 c.c. of cold saturated solution of ferrous sulphate, 1 c.c. of a watery or alcoholic solution of either fuchsin or methyl violet.

According to Loeffler this mordant is exactly suited for demonstrating the flagella of Spirillum concentricum. For typhoid, an addition of 1 c.c. of a 1% solution of caustic soda to 16 c.c. of the mordant is necessary, for B. subtilis 28–30 drops, and for B. mal. oed. 36-37 drops of the caustic. On the other hand, ½-1 drop of a solution of H_2SO_4 (exactly equivalent to the 1% solution of caustic soda) must be added to 16 c.c. of the mordant in the case of cholera and Spirillum rubrum.

According to Nicolle and Morax, neither caustic nor acid need be added if the preparation is treated in the mordant for three or four times in succession for ten seconds each time until steam is given off (not boiling), and carefully washed in water between each application of the mordant. Instead of being washed in alcohol as directed in 3 above, the preparation may be washed in water.

The formation of precipitate on the preparation can be avoided by very careful washing. To this end also it is practicable when applying the mordant or stain to cover the film with a small piece of blotting-paper, and to apply the fluid on the top of it.

Bunge's modification.

1. The following mordant is applied:—

3 parts concentrated watery solution of tannin, 1 part liquor ferri sesquichlor 1 : 20 aq.; to 10 c.c. of this mixture add 1 c.c. of a concentrated watery solution of fuchsin. This mordant should be kept for at least several days before use. Immediately before use peroxide of hydrogen is added drop by drop till the mordant assumes a reddish-brown colour (about 14 drops of 3 % H_2O_2 solution to 5 c.c. of the mordant are required). A few drops of the solution are then filtered on to the cover-glass and the latter warmed over the flame for five minutes. When H_2O_2 has been added to it, the mordant keeps for only a short time.

2. Wash in water.

3. Dry between blotting-paper.

4. Stain with carbol gentian violet (prepared in the same way as Ziehl's solution, p. 69 (*a*)), and heat gently.

5. Place for $\frac{1}{2}$-1 minute in 1% acetic acid. (May be omitted.)

6. Wash in water, dry, mount in balsam.

The above method can be applied to all bacteria without any addition to the mordant. Capsules are stained by it, especially if before being subjected to the mordant the preparation is placed for $\frac{1}{2}$-1 minute in 5% acetic and then washed in water.

The Modification of Coerner and A. Fischer.

1. Place the preparation in the following mordant, and heat for one minute without boiling:—Tannin 2 grams, water 20 grams, solution of ferrous sulphate of 1 : 2 strength 4 c.c., saturated alcoholic solution of fuchsin 1 c.c.

2. Wash in water.

3. Stain with aniline-fuchsin, or with carbol-fuchsin, or with saturated watery solution of fuchsin.

4. Wash in water. Dry. Mount in balsam.

This method is applicable for all bacteria without any addition to the mordant.

B. Van Ermengem's Method.

1. Place the cover-glass preparation for half an hour in the cold, or for five minutes at a temperature of 50-60° C., in a mixture of 1 part 2% osmic acid and 2 parts 10-25% tannin solution, to which mixture 4-5 drops of glacial acetic acid are added per 100 c.c. The mixture should, if possible, be a few days old.

2. Wash in distilled water.

3. Wash in absolute alcohol.

4. Immerse in 0·25–0·5% silver nitrate for a few seconds.

5. Without washing, place for a few seconds in the following solution:—Gallic acid 5·0, tannin 3·0, fused acetate of soda 10·0, aq. dest. 350 c.c.

6. Place the preparation back in 4, and keep it constantly moving till the solution begins to become black.

7. Wash in much water. If the staining is not sufficiently intense repeat 5–7; but if too dark, immerse in chloride of gold 1:3,000 for a moment, carefully wash, and expose the preparation for a few days to the light.

8. Dry with blotting-paper; mount in balsam.

Bacteria blackish-brown; flagella black.

The method may be applied to all motile bacteria in the same manner. Best carried out in bright daylight.

C. **Zettnow's Method** (*Klin. Jahrb.* Bd. XI, p. 379; older communication *Zschr. f. Hyg.*, Bd. 30, p. 95).

1. Preparation of the film by the osmic acid method (see p. 76).

2. Mordant. The cover-glasses are fixed in the flame, and laid face downwards in a 'block capsule', which is well filled with the mordant and placed for 5–7 minutes on an iron plate which is at a temperature of about 100° C.

Preparation of the mordant. Make a solution of 10 parts tannin in aq. 200, and warm it up to 50–60° C. Add 36–37 c.c. of a solution of 2 grams tartarus stibiatus (antimony tartrate) in 40 of water, and heat till the precipitate dissolves. If, on cooling, the mordant is very turbid (milk-white; test by pouring it into a test-tube) add some tannin solution; but if clear, add 1 c.c. of tartrate solution. The mordant should on no account form any precipitate, and it should be quite clear on heating. The addition of thymol ensures its preservation. The mordant should be applied warm and in clear condition.

3. Allow the capsules to cool down until the mordant begins to become turbid, then wash carefully with water.

4. On the cover-glass put 3-4 drops of ethylamine-silver solution, and heat until it fumes well and the edges of the film (these alone) become blackened.

Ethylamine-silver solution. 2-3 grams of silver sulphate are thoroughly mixed with 200 aq., in order to obtain a saturated solution. The desired quantity of this, together with an equal volume of water, is put into a test-tube and 33% ethylamine solution, added until the precipitate which forms is dissolved. The solution keeps well. The brown tinge which it gradually acquires is of no importance.

VI

Special Staining and Culture Methods for the most important Pathogenic Microorganisms and a few others

1. The Anthrax Bacillus.

B. anthracis grows on all ordinary culture media, liquefies gelatine, stains easily, and is Gram-positive. It is non-motile. Spore-formation occurs most quickly in the hot incubator, but does not take place below 16° C., or in the animal body (when whole).

The presence of B. anthracis in the body is proved by culture and by animal experiment (subcutaneous injection of mice and guinea-pigs). In the case of decomposed material the animal experiments sometimes fail, while plate cultures succeed. In the blood of infected animals which cannot be examined at once, the anthrax bacilli retain their vitality for some considerable time if the blood is dried in thick layer on glass. For demonstration of anthrax on hair, &c., wash the hair in alkaline broth and heat for half an hour to 80° C. (the anthrax spores survive), centrifugalize, and inoculate the sediment into animals and plates.

Johne's method of staining the capsule of B. anthracis in smear preparations of organs.

1. Stain in 2 % watery solution of methylene violet, applying moderate heat.

2. Wash in water for two seconds.
3. Wash in 1–2 % acetic acid for 6–10 seconds.
4. Wash in water and examine therein.

If the preparation is dried and mounted in balsam the capsules disappear.

The capsules may also be demonstrated by warming the preparation in a solution of Safranin (three grams of Safranin dissolved in 100 c.c. of almost boiling aq. dest.: when cooled, the solution is filtered). Another method of demonstrating capsules is by staining with gentian-violet (a cold, saturated solution of the dye in formalin; filtered). In this case the smear preparations are not fixed, and are subjected to the stain for thirty seconds in the cold. The preparations are examined in water.

The possession of a capsule sometimes enables B. anthracis to be distinguished from bacilli that resemble it in appearance, and are apt to occur in the animal body when decomposition has begun; but positive cultures and animal experiments are always also necessary before a definite decision can be given.

The Preparation of Spore-threads

Agar, or potato cultures in which the presence of spores has been proved by microscopical examination are broken up and emulsified in sterile water. Sterile silk threads (No. 4 or 5) 1–2 cm. long are soaked in this emulsion (or are rolled directly on the agar culture). They are then dried on sterile plates in the exsiccator, and preserved in sterile test-tubes in the dark. Handle the threads with care, owing to the danger of spore inhalation. The use of these threads for testing disinfectants is described on pp. 48, 49. As a rule they withstand steaming at 100° C. for 2–5 minutes.

2. The Tubercle Bacillus.

The usual media for B. tuberculosis are blood serum (best for cultivation from the body), glycerine agar (optimum 4 % glycerine), glycerinated potato (wedges, see p. 27, are boiled in water containing 4 % glycerine and the fluid then poured away), glycerine broth (growth occurs only in the form of particles floating on the surface). Cultures must be kept at a temperature of 37° C., and growth is very slow. Drying up of the medium is prevented by covering the culture tubes with a rubber cap. There is practically no growth on gelatine, peptone-agar, &c., or at room-temperature. The tubercle bacillus is non-motile.

Special media, giving good growth already after eight days, are Hesse's Heyden agar, p. 88, or Ficker's brain medium. The latter is prepared as follows:—

Finely chopped brain is mixed with an equal part of aq. dest., and warmed to boiling, constantly shaking. The mixture is boiled for a quarter of an hour, then strained to a thin pulp. With this is next mixed, without neutralizing, an equal part of 2·5 % agar dissolved in aq. dest.; 3 % of glycerine is then added and the whole sterilized. When the medium is poured into tubes, the tubes should be well shaken, and then set quickly before the layers of brain and agar have time to separate.

Methods of Staining the Tubercle Bacillus.

Tubercle bacilli stain with difficulty, but when once stained retain the stain very firmly. The following methods demonstrate only tubercle bacilli, and similar so-called acid-fast bacilli. As regards differentiation of the latter from tubercle bacilli, see p. 91. In these processes the tubercle bacilli take the primary stain, whilst other bacteria and the tissue elements take the counter-stain.

(*a*) The following two staining methods are most to be recommended :—

For films.

1. Stain with aniline water fuchsin, or with carbol fuchsin for two minutes, boiling repeatedly.
2. Decolorize for 2-5 seconds in 5 % H_2SO_4, or in 25 % HNO_3.
3. Wash in 70 % alcohol till the film appears colourless (if this does not occur quickly, repeat 2 and 3).
4. Counter-stain with either saturated watery solution of methylene blue, or with Loeffler's blue (p. 61), 1 part and 3 parts of water for 5-10 seconds.
5. Wash in water.

For sections.

1. Stain in aniline water fuchsin (carbol-fuchsin is not so good, because the preparations are less clean) for from fifteen minutes to twenty-four hours.
2. Decolorize for ten seconds in 5 % H_2SO_4, or in 20 % HNO_3.

blue 1, crystallized soda 0·2, aq. 100, for a few up to 20 seconds without applying heat. Wash in water. Parasites blue, red corpuscles greenish.

b. Double staining with Eosin and Methylene blue according to Plehn.

1. Stain in the following mixture for 5-6 minutes (warming slightly for one minute):—

60 c.c. saturated watery solution of methylene blue,
20 c.c. ½% eosin dissolved in 75% alcohol,
40 c.c. aq. dest.,
12 drops 20% caustic potash.

2. Wash in water.

Nuclei of the leccocytes dark blue, plasmodia bright blue, erythrocytes and granules of the eosinophile cells rose coloured.

c. Chromatin stain of Romanowsky-Nocht.

2-3 drops of 1% watery solution of eosin are diluted with 1-2 c.c. of water. To this is added drop by drop a solution in water of 1% methylene blue med. pur. Höchst, and ½% soda, which mixture must have been kept for two days at 50-60° C., or eight days at 37° C. After which it is used cold. The alkaline methylene blue solution is added until the eosin solution becomes so dark that no traces—or scarcely any—of the colour of the eosin are perceptible. On the surface of this mixture of stains the cover-glass preparations (which should not be more than a few months old at the outside) are allowed to float for 5-10 minutes. Wash in water, dry, mount in balsam. The stain is freshly mixed for each preparation. The chromatin of the malarial plasmodium is shining red, its protoplasm blue; leucocyte nuclei red to violet, their protoplasm blue; red blood corpuscles rosy to brownish-red. A modification of the method for old dried films has been described by Ruge, *Zeit. f. Hyg.*, Bd. 33.

d. Chromatin stain according to Giemsa (a modification of *c*). (*Centr. f. Bakt.*, Abt. 1, Or. 37, p. 308.)

1. Fix the preparations in ethyl alcohol for 15–20 minutes, or (only for 2–3 minutes) in methyl alcohol. Remove the fluid by blotting-paper.

2. Stain with Giemsa's solution for Romanowsky stain, which can be obtained from Grübler, Leipzig. Fill this stain into a drop-bottle (which before use must be rinsed out with absolute alcohol). To some water which has been warmed in a test-tube to 30–40° C. add, while it is being shaken, 1 drop of the stain for each cubic centimetre of the water. (The freshly diluted stain is run on to the cover-glass preparations and allowed to act from 10–15 minutes.)

3. Wash in running water.

4. Dry, and mount in balsam.

The staining resembles that seen in *c*. The method is applicable for staining sections and further for trypanosomes, piroplasma, &c.

VII

Methods of obtaining Material from the Body for Bacteriological Examination

(Revised and amplified by Dr. T. J. Horder, F.R.C.P.)

VARIOUS secretions, pathological exudates, and other fluids which are to be examined bacteriologically are removed and preserved in such a manner that (1) they do not become contaminated by extraneous micro-organisms, and (2) they do not come in contact with disinfectants.

(I) **Blood.** *A.* Small quantities of blood, such as are required for Widal's test or other agglutination reactions (see p. 122), the technique of phagocytic or opsonic indices (see pp. 170, 173), the investigation of bactericidal action (see p. 169), or the preparation of culture media, may be best withdrawn from the skin at the dorsal surface of the terminal phalanx of the finger. The finger is scrubbed with soap and water for some minutes and is then rubbed with sterile wool soaked in equal parts of absolute alcohol and ether. The cleansing process ensures disinfection of the skin and also induces a good flow of blood to the part. The blood flows more freely from the puncture if an elastic band is wound round the proximal part of the finger immediately before the puncture is made. This object is also ensured by swinging the arm well before the elastic band is used. It is also further ensured by flexing the terminal joint of the finger. When a small lancet or bayonet-pointed needle has been sterilized in the Bunsen flame, a little alcohol is poured over the finger and allowed to evaporate. The puncture is then made. If these directions be carried out a considerable amount of blood may easily be obtained. The blood is collected in capillary tubes, the sealed ends of which are broken off by sterile forceps and passed through the flame, to be sealed after the blood has been drawn. The size of the tube varies with the amount of blood which it is required to collect. The puncture wound may be closed by application of a little collodion.

If the *serum* is required, this is allowed to separate by standing the blood in the ice-chest; or its separation is promoted by centrifugalizing. The tube is then broken by sterile forceps, and the end of a fine glass pipette is introduced into the serum, which rises into the tube to any measured distance. From this tube the serum may be transferred to any required vessel, and, if necessary, diluted to any desired degree.

B. Larger quantities of blood, such as are required for purposes of *blood-culture*, are best obtained by puncturing a good-sized vein at the bend of the elbow with a sharp, clean hollow needle attached to a glass syringe capable of holding 5 or 10 c.c. Needle and syringe are boiled in distilled water immediately before use. The skin over the vein is cleansed as in *A*, the arm is allowed to hang down fully extended, and, if the vein is not already rendered prominent by this means, a bandage is tied round the

arm near the shoulder. It is advisable to draw up
the neck of the syringe a little of a 0·5 % steri
solution of sodium citrate to prevent clotting in
needle. The needle is then inserted into the vein
being taken to hold the syringe as far as po
parallel to the surface of the arm: by this
puncture of the opposite wall of the vein is av
If the needle is in the vein, the blood will ent
will often fill the syringe without suction
employed; no more suction than is necessary
the syringe in a reasonable time must be used
tends to introduce air at the junction of the
and syringe. This method of obtaining bloo
far the best when it is required for purp
determining the presence of micro-organism
because the blood at no time comes into cont
the skin or with the air. The syringe being
needle is quickly withdrawn, a finger being
upon the puncture wound whilst an assi
moves the bandage and raises the arm. Th
ture then ceases to bleed, and may be seal
a little collodion. For purposes of blood-cu
blood is now squirted into tubes of variou
and these are incubated at 37° C. The syrin
graduated, definite amounts of the bloo
added to broth tubes, or sown on solid m
thus a quantitative estimation of the o
present in the blood may be obtained. For
of preparing blood-media for the growth o
philous organisms, proceed as on p. 149.

C. Blood from the cadaver is withdrawn
heart by searing the surface of the

and inserting [illegible] te, or the needle [illegible] usly sterilised.

ake a blood [illegible] amination, [illegible] been sterilised [illegible] aced against [illegible] puncture wound. [illegible] the surface [illegible] are then [illegible] the layer of blood [illegible] thin. The [illegible] ng covered [illegible] ey are placed [illegible]

[illegible]

from the throat theria, &c.). With d, and the tongue with a sterilised erile wool fixed to). It is an advan- a sterilised tube, ting so that it can re obtainable from

In removing the that the affected hed—if any doubt ated. After [illegible] replaced in its tube, de forthwith. [illegible] aterial from drying [illegible] [illegible]. For exact acteria present and essential to make own amount of the of it). *Nasal* [illegible] er; the [illegible] [illegible] and a [illegible]

ed as free from [illegible] ould be [illegible]

[illegible]

points of the iliac crests is that of the fourth lumbar vertebra. The puncture is made midway between this spine and the one next above or below it. In an adult a point is chosen ½ inch, in an infant somewhat less than ½ inch, from the middle line. The direction of the needle in an adult is important: it should be forwards, inwards, and upwards. In infants the direction may be almost straight forwards. The needle is pushed firmly onwards until the gap between the laminae is entered, and fluid flows from the needle. If the course of the needle seems obstructed it must be withdrawn a short distance, any faulty direction corrected, and a fresh attempt be made. Suction by the piston is not required; if the needle is in the spinal canal and its orifice is patent any fluid present will flow, whether there be meningitis or not. A dry puncture may mean that the needle has not entered the canal, or that it has struck a nerve root, or that the pus present is too inspissated to flow. The first is the most usual explanation.

B. From the cadaver cerebrospinal fluid may be obtained by lumbar puncture, or it may be collected from the ventricles of the brain or from the distended pia-arachnoid membrane by sterile glass pipettes, which are sealed immediately afterwards.

(III) **Pus and various pathological exudates** are best withdrawn by a sterile syringe, after thorough disinfection of the skin. The syringe is taken to the laboratory, or the material is transferred from it to sterile test-tubes, or is sown directly into tubes containing sterile water, from which cultures may be made later upon appropriate media.

(IV) **Secretion or membrane from the throat** (Cases of sore throat, suspected diphtheria, &c.). With the patient's mouth well illuminated, and the tongue depressed, touch the diseased part with a sterilized platinum loop or with a plug of sterile wool fixed to a wooden rod or wire (i.e. a 'swab'). It is an advantage to keep these throat swabs in a sterilized tube, the handle of the instrument projecting so that it can be readily grasped. Such swabs are obtainable from most bacteriological laboratories. In removing the material, care must be exercised that the affected part of the throat is really touched—if any doubt arises, the operation must be repeated. After touching the part, the swab or loop is replaced in its tube, unless the examination can be made forthwith. Care should be taken to prevent the material from drying up, as some bacteria (e.g. meningococcus) rapidly perish under the influence of desiccation. For exact determination of the kinds of bacteria present, and their relative abundance, it is essential to make quantitative dilutions of a known amount of the material (e.g. a standard loopful of it). *Nasal secretion* is removed in a similar manner; the operation is facilitated by the use of a speculum and a head mirror.

(V) **Sputum** should be obtained as free from food and from saliva as possible. It should be expectorated directly into a clean vessel, which, of course, contains no disinfectant. In making the necessary examinations care must be exercised in selecting portions of the sputum most likely to give positive results. Thus, if the question of tubercle bacilli is under investigawhich this action appears to depend he found to be thermo-stable, specific and only present in immune bloods. Saturation experiments further showed that, when present, they could not be removed by the bacteria in question, as can be done in the case of opsonins.

Their presence may be demonstrated either by Leishman's original method or by Wright and Douglas's modification. In the former instance, a trace of the serum of an immunized person or animal —from $\frac{1}{25}$ to $\frac{1}{100}$ of the total volume of the mixture of blood and bacterial suspension—is added to the normal blood, the control specimen having a similar addition of a normal serum in order to make the results comparable. The phagocytic ratio is then ascertained in the usual way. When dealing with bacteria which are susceptible to the action of bacteriolytic substances in the immune serum it is well to heat the immune serum before adding it to the normal blood.

The modification of W. and D. may also be used. In this, the leucocytes of the blood are freed from the blood fluids by citration and a series of washings and centrifugings, the serum being finally restored at the moment of adding the bacterial suspension. The whole of the serum used in the estimation must be previously heated to 60° C. for 15 minutes in order to remove any thermolabile opsonin that may be present and, also, to obviate the under-estimation which may result in the case of a strongly bacteriolytic serum. The following mixtures are prepared :—

1. 'Control' tube.

Heated normal serum . . .	3 volumes.
Bacterial suspension . . .	1 "
Washed blood cells . . .	3 "
Heated normal serum ($\frac{1}{3}$ dilution) .	1 "

2. 'Stimulin' tube.

Heated normal serum . . .	3 volumes.
Bacterial suspension . . .	1 "
Washed blood cells . . .	3 "
Heated immune serum ($\frac{1}{3}$ dilution)	1 "

The difference in the contents of the two tubes lies in the addition to tube 2, containing heated normal serum, of a trace of the heated immune serum, the possible effects of this dilution of the serum being controlled in tube 1 by a similar addition of diluted and heated normal serum.

Films are then prepared in the usual way after incubation of the tubes for 15 minutes at a temperature of 37° C.; these are stained, the ingested bacteria enumerated in the polynuclear leucocytes and the average calculated. The 'stimulin' effect is recorded by dividing the phagocytic index of the 'stimulin' tube (No. 2) by that of the 'control' tube (No. 1).

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1. ‘Control’ tube.

Heated normal serum . . .	3 volumes.
Bacterial suspension . . .	1 ,,
Washed blood cells . . .	3 ,,
Heated normal serum ($\frac{1}{5}$ dilution) .	1 ,,

2. ‘Stimulin’ tube.

Heated normal serum . . .	3 volumes.
Bacterial suspension . . .	1 ,,
Washed blood cells . . .	3 ,,
Heated immune serum ($\frac{1}{5}$ dilution)	1 ,,

The difference in the contents of the two tubes lies in the addition to tube 2, containing heated normal serum, of a trace of the heated immune serum, the possible effects of this dilution of the serum being controlled in tube 1 by a similar addition of diluted and heated normal serum.

Films are then prepared in the usual way after incubation of the tubes for 15 minutes at a temperature of 37° C.; these are stained, the ingested bacteria enumerated in the polynuclear leucocytes and the average calculated. The ‘stimulin’ effect is recorded by dividing the phagocytic index of the ‘stimulin’ tube (No. 2) by that of the ‘control’ tube (No. 1).

IX

Inoculation and Post-mortem Examination of Animals

Inoculation.

HAIR or feathers, if they interfere with the inoculation, are removed, the skin is cleansed with soap, followed by alcohol, and sterile water.

1. *Cutaneous inoculation.* Either the skin is slightly incised and a platinum loopful of the material inserted into the wound, or the skin is scratched and the material well rubbed in.

2. *Subcutaneous inoculation.*

a. In a pocket of skin. A fold of skin is raised up with the forceps, a small opening made in it with sterilized scissors, and a small pocket made in the subcutaneous tissue either with the point of the scissors or with a sterilized lancet. Into this pocket the material is introduced by means of a platinum loop.

Rats and mice are inoculated preferably at the root of the tail. They are grasped by the neck with dressing-forceps, and the tail is held in the hand. Guinea-pigs are inoculated on the chest or abdomen, rabbits in the ear, and birds in the breast.

b. With the syringe. Different kinds of syringe are used for subcutaneous injection. Among the best are those of Pravaz (with asbestos plunger), Koch, Stroschein, or Loeffler (with easily improvised rubber plunger). The syringe must be sterilized before and after use, either in the steamer, or with alcohol and ether. Previous disinfection of the skin at the point of injection is unnecessary. The injection is made at the places mentioned above (guinea-pigs under the skin of the abdomen).

3. *Intraperitoneal injection.* The skin is incised and a canula passed through the muscular wall into the peritoneal cavity.

4. *Intramuscular, or intrapleural injections* are made as above.

5. *Injection into the blood-stream.* Expose an easily accessible vein (i. e. the external jugular), introduce a finely-pointed canula, and inject in the direction in which the blood is flowing (i. e centripetally). Care must be taken to avoid introducing air, or death may result from air-embolism. In rabbits the ear vein is generally made use of for this purpose, and prior to injection pressure is exerted on the root of the ear to cause the vein to enlarge. If, when the injection is made, the surrounding tissue is distended, it is a sign that the fluid is not being injected into the vein.

6. *Injection into the stomach.* A plug of wood perforated by a hole is inserted between the front teeth, and through the hole in this plug a rubber catheter is passed into the stomach. The material is then poured down the catheter into the stomach. In the case of larger animals the material may be introduced into the stomach by enclosing it in pieces of potato. A cavity is made in a small piece of potato, filled with the material, and closed over with potato. The piece

of potato is then pressed back against the pharynx of the animal so that it is swallowed. Infected pieces of bread may be made use of in the same way.

7. *Infection by feeding.* When the infected material is not in itself nutritious it can be administered mixed with some food (e. g. a culture smeared or powdered on bread).

8. *Infection by the air passages.* This is effected by causing animals to inhale, in a tightly-closed inhalation apparatus, air contaminated with bacteria, or with infected dust. Or the material may be injected directly into the trachea.

Dosage of Inoculation Material.

Quantitative injections are effected in the following ways:—

1. *Fluid materials.* The quantity decided upon is measured out, dilution with sterile broth or with 0·8% NaCl solution being resorted to if necessary for this purpose, and the injection is then made with a graduated syringe.

2. *Solid materials.* For removal of the material, use may be made of a platinum loop at the end of a short platinum wire, the other extremity of which is fastened to a handle by a screw arrangement (like a certain form of pocket pencil). This detachable platinum loop is first stuck into a piece of cork and weighed, then charged with the inoculation material and reweighed. The difference between the two weighings gives the weight of the inoculation material attached. The contents of the loop are then distributed in a definite quantity of sterile fluid in such

manner that an easily measured quantity of the suspension (e.g. 0·5 c.c.) contains the quantity of the material which it is desired to inoculate, and after thorough shaking, this quantity is then injected. By using the same loop, the same inoculation material, and by filling the loop with as far as possible the same amount of it, equal results are obtained, and the weighing may be dispensed with. To this end a series of loops should be kept in stock of such size that they require about 2 mgr. of twenty-four hours' agar culture to completely fill them—so-called Normal Loops. Measurements for the preparation of loops of standard size have been given by Czaplewski.

Determination of the Number of Bacteria in the Dose of Material Inoculated.

The material is withdrawn from the culture as described above, and a quantity of the suspension equal to that used for injection is sown on agar or gelatine plates. If desirable, the suspension is first diluted with a measured quantity of sterile broth or 0·8% salt solution, and an aliquot part of this dilution sown. The colonies that develop are counted as in the case of plate cultures from samples of water (p. 195). In a similar manner the bacteria in all kinds of material may be estimated.

Preservation of Experimental Animals.

Mice and rats can be kept in labelled glass boxes or jars with wire covers, and guinea-pigs in stone pots with wire tops labelled with their weight, sex, and distinctive markings. Special diagrams of the outline gelatine has dried, it is covered over with a layer of varnish.

4. One heats a metal compound of low melting-point (e.g. Rose's metal, fusible metal, &c.) in a Bunsen burner, and allows a drop of it to fall from a height of ¼-½ meter on to a glass plate. The flat metal disk thus produced from the drop is applied to the mouth of the tube after removal of the rubber cap, its overlapping edge is pressed firmly down round the rim of the tube, and the latter is hermetically sealed by gently rotating it and at the same time warming it over the Bunsen burner.

5. The rubber cap and plug of wool are removed and the tube fused a short distance below its orifice. Take care that the medium is not impaired by the heat.

N.B.—Tubes which contain condensation water are always kept in a horizontal position, or this water is carefully poured off before treatment with formalin.

Plate cultures may be preserved as follows:—

1. Two drops of formalin are placed on the inside of the cover, and the plate is allowed to stand for twenty-four hours. Whenever practicable, the plate should be inverted so that the formalin does not drop on to the medium; and a good method is to fasten a piece of blotting-paper on the cover of the plate, and to moisten this with the formalin. If at the end of twenty-four hours the formalin has not all evaporated, the remains of it are removed and a broad rubber band placed over the edge of the plate.

2. The culture is made on plates of which the cover is considerably larger than the other half of the plate.

After growth has taken place the plate is then treated with formalin as in 1, and sealed off with melted paraffin at the point of contact.

3. The culture is made in a flat flask, shaped like a flattened test-tube, and dealt with as directed in the case of tube cultures.

Portions of agar and gelatine cultures can be preserved in the following ways:—

1. Treat with formaldehyde as directed above. Carefully excise the parts to be preserved, and transfer them to slides (or dry over H_2SO_4 till only a thin layer remains). Cover with glycerine and with a cover-glass, and ring with asphalt-varnish. The appearance of the colonies is impaired by this treatment, and the method is only applicable for agar plates.

2. Make a plate culture on a cover-glass, and after development has taken place, dry over H_2SO_4. Stain as in case of a dry preparation, and mount in Canada balsam. (Not suitable so much for inspection of colonies as for study of the arrangement of bacteria in them.)

c. Preservation of organs of animals for purpose of demonstration.

1. Place the organs in the following solution until completely decolorized:—Water 4,000, formalin 800, potassium acetate 85, potassium nitrate 45.

2. After allowing the fluid to drain off, place in 80% alcohol till the natural colour returns.

3. Preserve permanently in the following solution:—aq. dest. 900, glycerine 300, potassium acetate 200 (Strassmann's method).

XI

Bacteriological Examination of Water, Milk, Shellfish, Vegetables, Sewage and Sewage Effluents, and Soil and Excremental matters

(Revised and amplified by Dr. A. C. Houston, Director of Water Examinations, Metropolitan Water Board).

General Remarks. The usual object in making a bacteriological examination of water, milk, soil, &c., is to determine the quality thereof in relation to undesirable pollution. With this end in view, the full bacteriological examination is of threefold description; attention being directed towards ascertaining (1) the total number of bacteria present per gram or cubic centimetre of the material; (2) the relative abundance of certain definite bacteria significant of pollution; (3) the presence or absence of certain specific pathogenic bacteria. In all three cases the examination should invariably be quantitative.

Total number of bacteria and number of spores of bacteria. This is usually estimated by cultivating a known quantity of the material in gelatine plates at 20–22° C. The number of colonies is counted usually on the third day. The number of bacteria capable of developing at 37° C. can be determined by agar plates made in the same way, the colonies being counted usually on the second day. If it is desired to estimate the number of spore-bearing bacteria present, a known quantity of the material is heated to 80° C. for ten minutes before being submitted to cultivation. The ratio between these three tests is often of importance. If a series of dilutions have been made, it is possible to form an estimate of the total number of micro-organisms present by inoculating tubes of fluid media (e.g. broth tubes) with falling quantities on the decimal scale, and by observing the dilution at which growth is no longer positive (e.g. 10,000, not 100,000 bacteria per c.c.; &c.).

Bacteria significant of pollution. Excremental material is the usual form of pollution for which substances of the above description are examined; and the index bacteria estimated are B. coli, streptococci, and spores of B. enteritidis sporogenes (Klein). It has been shown (L.G.B., 1902-3) that normal human excrement contains these bacteria to the following extent per gram:—B. coli (flaginac) about 100 million; streptococci at least 100,000; spores of B. enteritidis sporogenes about 1 million. It has also been found (second and fourth reports R.S.C.) that ordinary sewage contains these bacteria to the following extent per c.c. B. coli about 100,000 (over 75% of them flaginac); streptococci at least 1,000; spores of B. enteritidis sporogenes 100 to 1000. The foregoing figures are only approximate. By the relative abundance, therefore, of these index bacteria in a given sample, the amount of faecal contamination contained by it can approximately be measured. B. coli is usually estimated by inoculating the glucose bile-salt medium of MacConkey with measured quantities of the material under examination. Streptococci can be estimated either by inoculating broth tubes in the same way and examining their growth microscopically (Gram-stained specimens) after incubation for 48 hours at 37° C., or by making surface cultures on plates of agar or Drigalski and Conradi's medium and subculturing the smallest colonies that develop. Spores of B. enteritidis sporogenes are usually estimated by inoculating milk in similar fashion and then incubating this milk at 37° C. anaerobically after subjecting it to a temperature of 75° C. for 10 minutes in a water bath. If the enteritidis change takes place in the milk, the result is recorded as positive.

It is necessary to isolate the Cóli and streptococci in pure culture and to test their characters—the Coli in the flaginac tests, and the streptococci in the differential tests described above (p. 142). In the case of the enteritidis test the characteristic change in milk is sufficient for ordinary purposes; but if the case is a particularly important one, 1 c.c. of the whey should be injected into a guinea-pig and its pathogenicity tested.

Specific pathogenic bacteria. Special methods for isolating the typhoid bacillus and the cholera vibrio have been described elsewhere (pp. 125, 133). The presence or absence of anthrax, tetanus, or tubercle, can be determined by direct inoculation of animals with definite amounts of the material. Other pathogenic bacteria require special methods for their isolation.

Water.

Collection of sample. In the case of wells provided with pumps, samples are collected in sterile bottles after pumping for some time. In case of open wells, springs, streams, &c., the sample is collected in a sterilized bottle by hand if possible, or by lowering a weighted bottle with a cord; or it is collected by means of one of the special apparatus designed for this purpose (e. g. Esmarch's flask, which is closed by means of a weight covered with rubber, and can be opened and closed at any depth; or Sclavo's so-called 'smash bottles' consisting of a test-tube drawn out into a thin bent tube with fused extremity: when the tube has been sunk to the desired depth its drawn-out extremity is punctured by means of a weight which travels down a long cord, and the tube then rapidly fills with water).

Preservation of samples. In cases where it is impossible to examine the samples at once, they are packed in ice.

Enumeration of general bacterial content. The number of bacteria present per c.c. of the sample can be estimated by making gelatine plates from 1 c.c., 0·1 c.c., 0·01 cc., and 0·001 c.c. of the water; the last three amounts being obtained by the dilution method.

If desirable, the number of bacteria capable of developing at 37° C. can also be estimated by making agar plates in the same way.

The Bacteriological examination of Water for Excremental Pollution.

1. The B. coli Test.

Stage 1. *Decimal mode of dilution.*

Add 9 c.c. of water to each of a number of test-tubes (6″ × ¾″), plug and sterilize.

Label six of these tubes (1), (2), (3), (4), (5), (6).

(*a*) Add 1 c.c. of the water to be examined to tube 1 and shake.

(*b*) Add 1 c.c. from tube 1 to tube 2 and shake.
(*c*) „ „ 2 „ 3 „
(*d*) „ „ 3 „ 4 „
(*e*) „ „ 4 „ 5 „
(*f*) „ „ 5 „ 6 „

The values of the above dilutions may be expressed as follows :—

(*a*) 1 c.c. of dilution 1 = 0·1 c.c. of original water.
(*b*) „ „ 2 = 0·01 „ „
(*c*) „ „ 3 = 0·001 „ „
(*d*) „ „ 4 = 0·0001 „ „
(*e*) „ „ 5 = 0·00001 „ „
(*f*) „ „ 6 = 0·000001 „ „

In the case of grossly impure waters it is necessary to make these six dilutions, but with potable waters it is seldom necessary to go beyond dilution (2). For purposes of description it will be assumed that only dilutions (1) and (2) have been made.

Stage 2. *The making of primary Cultures.*

Add 1 c.c. from dilution (2) to a test-tube (6″ × ¾″ with an inverted inner tube 2″ × ½″) containing

10–12 c.c. of single strength bile-salt glucose peptone water.

Deal similarly with dilution (1); then add 1 c.c. of the undiluted water to a third tube of the same medium.

Now add 10 c.c. of the water to a fourth tube containing 10–12 c.c. double strength bile-salt glucose peptone water.

Lastly, add 100 c.c. of the water to a fifth large tube (8″ × 1½″ with inverted inner tube 3″ × 1″) containing 50 c.c. of treble strength bile-salt peptone water.

Stage 3. *The making of secondary Cultures.*

Withdraw a loopful from the primary tube and add it to a test-tube containing 10 c c. of sterile water. After shaking, withdraw a loopful from the latter tube and smear it over the surface of an oblique, neutral red bile-salt agar tube, or a plate into which this medium has been poured. Incubate at 37–42° C. for 24 hours. B. coli shows as bright red colonies, and one or several of these colonies should next be subcultured into melted glucose gelatine and 'shake' cultures made. B. coli and like forms produce gas in this medium within 24 hours at 20° C. If gas formation in glucose is positive, the biological attributes of the microbe must be further studied.

Stage 4. *Determination of the type of B. coli.*

Many tests are available for this purpose, but a useful combination is the Flaginac test.

Four tubes containing different media are inoculated from the glucose gelatine culture, namely :—

Neutral red broth culture (fl = fluorescence within two days at 37° C.).

Lactose peptone culture (ag=acid and gas within two days at 37° C.).

Peptone water culture (in=indol within five days at 37° C.).

Litmus milk culture (ac=acid and clot within five days at 37° C.).

A micro-organism yielding positive results with all these four tests is described as a flaginac B. coli, that is, a microbe indistinguishable on the basis of the tests employed from typical B. coli of the alimentary tract.

Modifications.

Stage 2. Other media may be employed, e. g. ordinary broth, bile-salt lactose peptone water, phenolized broth, peptone water, &c.

Stage 3. Other media may be used, e. g. Drigalski and Conradi, ordinary agar, phenolized agar, ordinary gelatine, phenolized gelatine and various sugar agars.

Stages 3 and 4. A useful modification is the Quintuple Preferential B. coli test described in the Report on the Metropolitan Water Supply for January, 1907. Its description here would occupy too much space.

Stage 4. Additional tests may be used, for example: motility; Gram's stain; nitrate broth; growth on various solid media; reactions with various sugars and alcohols such as saccharose, raffinose, laevulose, mannose, dextrin, dulcit, isodulcit, mannite, salicin, &c.; Proskauer's and Capaldi media, &c. MacConkey has recently recommended Vosges and Proskauer's reaction and the following substances as having special differential value—lactose, saccharose, dulcit, adonit, and inulin.

It may here be added that in Stage 2, surface cultures on solid media may be used instead of liquid including the reactions in the various differential tests described previously (p. 143).

3. THE SPORES OF B. ENTERITIDIS SPOROGENES TEST.

For the purpose of dilution, stage 1 of the B. coli test may be used. For larger volumes of water than 10 c.c., concentration of the bacterial contents of the water is necessary. The medium employed is 'whole' milk, and after inoculation with the water, the culture is heated to 80° C. for 10 minutes, and then incubated under anaerobic conditions at 37° C. After two days' incubation, the results are recorded; if the casein is precipitated and torn into irregular masses with a moderately clear whey and evolution of gas, the result is considered positive. But it is desirable to verify by animal inoculation and by various cultural tests.

There are many other tests which may be usefully employed in the bacteriological examination of water, e.g. determination of the ratio between the number of microbes growing respectively at 20° C. and at 37° C.; enumeration of the spores of aerobic bacteria; animal inoculation tests, &c. But these cannot be considered here. Similarly, special tests for specific pathogenic bacteria do not fall within the province of the present article.

The following media are used in connexion with the above method of examining water.

MacConkey's Bile-salt glucose peptone water.

Peptone 20 grams, glucose 5 grams, taurocholate of sodium 5 grams, 100 c.c. of a 10% sterile litmus solution, tap-water to 1 litre. Put peptone, glucose, taurocholate of sodium, and water in a flask, and heat in steamer for 45 minutes. Filter through Chardin's filter-paper, add the filtered litmus solution and place in tubes (with Durham's fermentation tubes). Steam for 45 minutes on two successive days.

Double strength bile-salt glucose medium.

The above solid constituents (peptone, glucose, taurocholate) are doubled. The medium is put into tubes in quantities of 10 c.c. The water to be examined is added to the medium in quantities of 10 c.c.

Triple strength bile-salt glucose medium.

The solid constituents are used in threefold strength. The medium is put into large tubes in quantities of 50 c.c. The water to be examined is added in quantities of 100 c.c.

MacConkey's Neutral red bile-salt agar.

Agar fibre 20 grams, peptone 20 grams, lactose 10 grams, taurocholate of sodium 5 grams, 4 c.c. of a 1% sterile aqueous neutral red solution, tap-water to 1 litre.

Soften agar in acidulated water as previously described. To the washed agar add peptone, bile-salt, and 500 c.c. tap-water. Heat in autoclave for 30 minutes, or in steamer for 90 minutes, then add remainder of water, cool to 60° C., add the beaten white of one egg, heat in autoclave for 45 minutes or in steam sterilizer for 90 minutes. Filter through a moistened Chardin's filter-paper, placing the funnel in a warm filter-jacket. When filtered, heat in steamer for 15 minutes, add lactose and neutral red, put in

tubes, and steam for 30 minutes on two successive days. The medium requires no alkali.

Peptone litmus water

(used for sugar tests, with reference to B. coli).

Sugar 1%, peptone 20 grams, 100 c.c. of a 10% litmus solution, tap-water to 1 litre.

Milk.

For details as to the bacteriological examination of milk in relation to standards, the reader is referred to a recent report (A. C. H.) to the L. C. C. (King & Sons: 1905).

Sediment. On p. 27 of the report in question, a convenient method and apparatus are described and figured by means of which the volume of dirt deposited by a definite quantity of the milk can be concentrated and measured, and known quantities of this deposit submitted to microscopical examination. The cover-glasses are 'flamed', immersed in ether for 5 minutes, transferred directly to absolute alcohol for a few seconds, and, without washing, placed film downwards in freshly prepared methylene blue solution (6 c.c. of the filtered saturated alcoholic solution of methylene blue, in 20 c.c. of a 1 in 10,000 solution of caustic potash) for not more than 10 seconds. After washing and drying, the preparation is mounted in Canada balsam in the ordinary way and is then examined with a one-sixth objective and a four ocular, the tube being drawn out to such an extent as to give a magnification of 460 diameters. Higher magnifications are necessary for special purposes. At least five

'fields' from each quarter of the cover-glass should be carefully examined.

Separate cover-glass preparations may also be stained as for tubercle, in the following manner:—

After treatment with ether and alcohol, the preparation is stained for several minutes in hot carbol fuchsin, washed, decolorized in 33% nitric acid, washed and counter-stained with methylene blue. After washing, drying, and mounting, the preparation is examined for the presence of acid fast bacilli, with a one-tenth or one-twelfth oil immersion lens, in place of the one-sixth objective previously recommended. As already pointed out, failure to find acid fast bacilli affords no convincing evidence of their real absence. Moreover, not all acid fast bacteria are tubercle bacilli (see p. 91).

The cultural procedure used was as follows:—

Dilution. The decimal mode of dilution should be carried out as described under Stage 1 of the B. coli test under *Water* (p. 196).

B. coli Test.

Primary liquid cultures and secondary plate cultures method.—The bile-salt glucose peptone medium of MacConkey may be used for the primary cultures, and, for secondary 'plating' purposes, the solid media recommended under bacteriological examination of water for excremental pollution (see above). The procedure is as follows:—

Ten c.c. of the milk are added by means of a sterile 10 c.c. pipette to a tube containing 10 c.c. of double strength bile-salt glucose peptone medium.

A sterile 1 c.c. pipette is used for inoculation from

tubes, and steam for 30 minutes on two successive days. The medium requires no alkali.

Peptone litmus water

(used for sugar tests, with reference to B. coli).

Sugar 1%, peptone 20 grams, 100 c.c. of a 10% litmus solution, tap-water to 1 litre.

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B. coli Test.

Primary liquid cultures and secondary plate cultures method.—The bile-salt glucose peptone medium of MacConkey may be used for the primary cultures, and, for secondary 'plating' purposes, the solid media recommended under bacteriological examination of water for excremental pollution (see above). The procedure is as follows:—

Ten c.c. of the milk are added by means of a sterile 10 c.c. pipette to a tube containing 10 c.c. of double strength bile-salt glucose peptone medium.

A sterile 1 c.c. pipette is used for inoculation from

the various dilutions in *reverse* order, i.e. 1 c.c. from dilutions (6), (5), (4), (3), (2), and (1), is successively added to a series of bile-salt glucose peptone tubes.

Finally, with the same pipette 1 c.c. of the undiluted milk is added to another bile-salt glucose peptone tube.

These cultures representing from 10 c.c. to one-millionth of a cubic centimetre of the milk are next incubated at 37° C. for two days.

All the primary cultures showing acid and gas formation are next subcultured in the way fully described under *Water*.

Primary plate culture method.—A gelatine medium or an agar medium (with or without the addition of one or another sugar) may be employed. But there is convenience in using the Drigalski and Conradi medium as this is specially suited for the isolation of streptococci, and also of the non-lactose-fermenting group of B. coli.

The procedure in any case is the same. The plates are inoculated with 0·1 c.c. of the various dilutions, and the material spread over the surface of the plates by means of a suitable sterile instrument. The plates are incubated at 37° C. and after 24–48 hours carefully examined. Selected colonies are subcultured in various media in the manner already described.

Streptococcus test.

As previously explained, the Drigalski and Conradi medium is well adapted for the purpose in view. The plates are inoculated with 0·1 c.c. of the various dilutions and the material spread over the surface of the plates by means of a suitable sterile instrument.

After incubation for 24-48 hours at 37° C. the minute colonies are subcultured in broth. The broth cultures are incubated for 24-48 hours at 37° C. and then examined microscopically. If the results of the microscopic examination prove satisfactory, the streptococcus is subjected to the differential tests for streptococci described above (p. 143) and also to the nitrate broth test (for observation as regards *absence* of ability to reduce nitrate to nitrite).

An alternative method is to inoculate a series of lactose peptone (+Lemco) tubes from the various dilutions. These tubes are incubated at 37° C. for 24-48 hours. As the great majority of milk streptococci produce acid in a lactose medium, only those tubes which show an acid change need be studied. Microscopic preparations from the tubes showing development of acidity are next made to see whether streptococci, mixed or unmixed with other bacteria, are present. If streptococci are present, secondary plate cultures must be made and the microbes studied in pure culture. This method works fairly well if the streptococci are present in large numbers compared with other microbes. But under these conditions almost any method would yield satisfactory results.

B. enteritidis sporogenes test.

The following amounts of milk should be used for cultural purposes:—100 c.c.; 10 c.c.; 1 c.c.; ·1 c.c.; ·01 c.c.; ·001 c.c. The 100 c.c. of milk is added to a large sterile tube; the 10 c.c. of milk to a large tube containing 30 c.c. of sterile milk, and the rest of the amounts to ordinary tubes containing 15 c.c. of sterile milk. The cultures are heated after inoculation, to 80° C. for ten minutes for the lesser amounts, and 15–20 minutes for the larger amounts of milk. The cultures are incubated, under anaerobic conditions, at 37° C. and observations made as regards the 'enteritidis change' after two days' incubation.

Shellfish.

The following method of examining oysters bacteriologically is taken from a Report (A.C.H.) to the Royal Commission on Sewage Disposal (Fourth Report, vol. 3; also *Journal of Hygiene*, vol. 4, no. 2, p. 185). The directions given apply, with modifications, to the examination of shellfish in general.

Cleansing of the Oysters:—

The outside of the oyster shell is well scrubbed with soap and water, and cleansed as thoroughly as possible under clean running water; the shells are then well washed in running *main* water, and finally with sterile water.

Cleansing of the Hands:—

The hands of the experimenter are thoroughly cleansed with a hard scrubbing brush, soap and water, then rinsed first with 1 in 1,000 corrosive sublimate solution and finally with sterile water.

Subsequent procedure:—

The oysters are laid out upon a sterile towel, the flat shell uppermost. They are opened in this position with a sterile knife, held in the right hand, while they are held in position with a corner of the sterile cloth grasped in the left hand. Great care is taken to avoid any loss of the liquor. This liquor in the shell is poured into a sterile 1,000 c.c. cylinder, the oyster is then partly cut up with sterile scissors, and the liquor thus freed also allowed to run into the cylinder; finally, the oyster is cut up into small pieces and added to the cylinder. Ten oysters are thus treated in each experiment. The volume of oyster + oyster liquor is read off, and usually varies between 80 and 120 c.c., so that the oysters, being of medium size and containing a medium amount of liquor, 100 c.c., might be considered a fair average of the total shell contents of ten oysters. Sterile water is then poured into the cylinder up to the 1,000 c.c. mark, and the whole well stirred with a sterile rod.

The following amounts of this liquid are taken for cultural purposes (primary cultures):—

Culture A 100 c.c. = contents of 1 oyster
,, B 10 c.c. = ,, ,, $\frac{1}{10}$,,
,, C 1 c.c. = ,, ,, $\frac{1}{100}$,,
Culture (1) 1 c.c. of $\frac{1}{10}$ dilution = $\frac{1}{1000}$ oyster
,, (2) 1 ,, $\frac{1}{100}$,, = $\frac{1}{10000}$,,
,, (3) 1 ,, $\frac{1}{1000}$,, = $\frac{1}{100000}$,,
,, (4) 1 ,, $\frac{1}{10000}$,, = $\frac{1}{1000000}$,,

These amounts—A, B, C, (1), (2), (3), (4)—are used for examination for B. coli (primary cultures), and the amounts B. C. 1, 2, 3 for the B. enteritidis sporogenes test. Experience has shown that it is best to make the primary cultures in triplicate. Then if, as regards the B. coli test, a sugar medium is employed, at least two out of the three primary cultures should form acid and gas to allow of a preliminary numerical diagnosis being made. Similarly, in respect of the B. enteritidis coli-like microbes, of any kind. On the other hand, the occurrence of gas in the primary cultures constitutes the *prima facie* or presumptive test for B. coli, and indicates the necessity for making from the liquid secondary cultures on solid media. As regards these secondary cultures reference should be made to stage 3 of the bacteriological examination of water for excremental pollution.

Reverting to the stage of the process at which the primary cultures representing the first 'washings' of the mixture of cress and water have been made: The next step is to pour off the remaining water in the flask, to add fresh clean water, shake the flask, then pour off the water, again to add fresh water and again to pour off the water. Repeat this procedure several times; then add to the flask sterile water, shake the flask and pour off the water. This should be repeated several times. Finally, add 900 c.c. of sterile water to the flask, shake it repeatedly, and proceed on exactly the same lines as before to examine the liquid (called the final 'washings'). During the process of washing, any simple device, such as holding a piece of sterile wire gauze over the mouth of the flask will prevent loss of small floating pieces of cress.

As the final 'washings' liquid is examined in exactly the same way as the first washings, it is unnecessary to repeat what has been already described.

Mention has only been made so far of the B. coli test, but of course the first and final 'washings' of the cress may be examined by the streptococcus, B. enteritidis sporogenes, or other tests.

Known quantities of the cress may also be added directly to primary liquid cultures, the subsequent procedure being on the lines already indicated.

Sewage and Sewage Effluents.

The method by which these materials are examined bacteriologically is similar to that practised in the case of water, but in the present instance dilution is carried further.

Detection of specific pathogenic bacteria in sewage is not easy by cultural methods at present available, and so far the animal experiment would appear to have afforded the best positive results. By this method Houston succeeded in demonstrating anthrax in the coke beds and septic tank sludge at the sewage works of a town where a number of tanneries are situate; and he further showed the presence of this same specific pathogenic micro-organism in mud of a stream contaminated by sewage from the same town (*Second Report*, R.S.C.). Among other pathogenic micro-organisms that have been recovered from sewage and sewage effluents are B. pseudo-tuberculosis and B. pyocyaneus.

Soil.

A platinum or metal spoon may be used for collecting a sample of the soil to be examined.

When examining the deeper layers of soil, either a hole is dug and samples taken from its sides at the required depth and inoculated, or Fraenkel's borer is used. The latter is driven into the earth by rotating it in a certain direction, and when the desired layer is reached, it is twisted round several times in the opposite direction, whereby a chamber in the point of the borer is opened and filled with earth. Then one draws the instrument out by rotating it in the first direction.

Examination of soil for excremental pollution. A definite quantity of the soil (e.g. a gram or more) is ground as finely as possible by means of a sterile pestle and mortar, or else is shaken up in a flask with sterile shot, &c., and is then diluted with known quantities of sterile water: the decimal mode of dilution being preferable. Cultures are then made from definite quantities of these dilutions, and the number of B. coli, streptococci, spores of B. enteritidis sporogenes, determined per gram of the original material. The characters of the B. coli and streptococci isolated should be determined in detail by the tests described elsewhere. For further details as to the bacteriological examination of soil for excremental pollution, see Reports (A. C. H.) to the Local Government Board, 1899–1900, ib. 1900–1, ib. 1901-2, also Savage, *Journal of Sanitary Institute*, 1903.

Specific pathogenic bacteria. When examining samples of soil for infective micro-organisms (tetanus, malignant oedema, anthrax, tubercle), one injects animals subcutaneously, either with some of the material itself or with some 0·8% NaCl solution with which the material has been shaken up.

Excremental Matters.

(e.g. faeces, cow dung, &c.)

The reader is referred to reports (A. C. H.) to the Local Government Board on this subject. But practically the same methods may be used as those described for liquids, except that it is necessary before making the various dilutions to thoroughly mix a known weight of the solid with a known volume of water.

XII

The Bacteriological Examination of Dust and Air

(Revised and amplified by Dr. Gordon).

Dust.

IF in sufficient quantity, the dust may be collected with a sterile knife and transferred to the laboratory in a sterile test-tube. Dust may also be collected from the air by exposing a sterile glass box, a sterile enamelled tray, or, if within doors and under a grating, a piece of highly glazed paper.

Examination for excremental pollution. A gram, or if possible, more, of the dust is weighed out, well crushed up in a sterile mortar with a little sterile water, and diluted up to a bulk of 100 c.c. for the primary dilution. From this a series of decimal dilutions are made through successive flasks as described above for milk. A series of cultures are then made in gelatine (22° C.) or agar (37° C.) for total numbers; in MacConkey's fluid for B. coli; in broth or on Drigalski and Conradi's medium for streptococci; and in milk for the spores of B. enteritidis sporogenes; the last cultures being heated to 75° C. for 10 minutes before incubation anaerobically at 37° C. The B. coli and streptococci are isolated in pure culture and their characters examined.

The result of some analyses of dust made on the above principle are seen in this table. The figures refer to the number approximately per gram of dust.

Source.	No. of Bacteria (37° C.).	B. coli.	Strepto-cocci.	B. enteritidis sporogenes.
Window bars and stonework at air inlet, House of Commons.	under 10	under 10	under 10	under 10
Week's sample of dust arrested from incoming air by scrim-cloth screen.	10,000	100	under 100	1000
3-months' sample of material falling through gratings in floor of Debating Chamber from boots.	100,000	100	10,000	1000
Dust of Debating Chamber.	100,000	1000	10	1000
Dust of Division Lobbies.	1,000,000	1000	1000	1000
Dust of New Palace Yard (cabs present).	100,000	10,000	under 1000	100

Air.

The following methods enable an estimate to be formed of the number of bacteria contained by the air.

1. *Hesse's method.* A glass tube 70 cm. long and 5 cm. wide is used. Gelatine is either spread all over the interior in the manner of a roll culture, or when, as is generally the case, the tube is used in a horizontal position, it is sufficient if a layer of gelatine 5 mm. deep is solidified on the floor of the tube throughout its whole length. The inlet end of the tube is provided with a rubber cap pierced by a hole, and to the opposite end of the tube an aspirator is attached by means of which air is slowly (10 litres in twenty minutes) drawn through. Tube, rubber cap, and gelatine are previously sterilized. Even in case of air comparatively free of micro-organisms it is advisable not to draw through more than 100–200 litres, otherwise the colonies lie too closely together. In the end of the tube to which the aspirator is attached (outlet end) a plug of sterile wool may be placed, and when the air has been drawn through, this plug may be sown in a gelatine plate. If the air has been drawn through sufficiently slowly, this plug remains free of bacteria.

2. *Petri's method* enables the micro-organisms to be collected from a larger quantity of air. By means of a pump with automatic counter attached, a measured quantity of air is drawn through two sand-filters, which are contained within the same glass tube and are separated from each other and from the outside

by small disks of metal gauze. The whole is of course sterilized before use. After the air has been drawn through, the sand and gauze are sown in gelatine or agar plates. If the filters are firmly pressed together, all the micro-organisms are held up in the first one. Instead of sand, pulverized sugar may be employed (it is, however, difficult to sterilize). Also grains of glass measuring from 0·25 to 0·5 mm., and contained in tubes of particular construction, may be used as a filter (Ficker, *Zeit. f. Hyg.*, Bd. 23).

3. The following method of *Bujwid* has the merit of simplicity. Air is slowly drawn through three wash-bottles communicating with each other, with rubber stoppers, and glass tubes, and each containing 2–3 c.c. of sterile water. The air yields up its micro-organisms to the water, various quantities of which are at the end worked up with gelatine.

4. *Frankland's method* (*Phil. Trans.* 1887, No. 2, p. 127). A known volume of air is drawn through a short piece of glass tubing constricted at two places to support plugs made of powdered glass wool mixed with sugar. The first plug holds up the micro-organisms, and cultures made from the second should remain sterile. This method has superseded Hesse's method.

5. *Method of Straus and Wurtz* (*A. I. P.* 1888, p. 171). A measured quantity of air is drawn through a special piece of glass apparatus containing 10 c.c. of sterile melted gelatine. The fluid retains the micro-organisms, and cultures are made subsequently from measured amounts of it. A drop of sterile oil is placed on the top of the fluid to control bubbling.

Broth may be used in place of gelatine, if desired. Air may be drawn through at the rate of 50 litres in fifteen minutes. The method is asserted to be more accurate than the others mentioned above.

Examination of Air for the Presence of Particulate Contamination.

The air of inhabited rooms is specially liable to derive particulate pollution from the presence of droplets of mucus sprayed from the mouth in the act of sneezing, coughing, and loud speaking (Flügge); from particles of epidermis shed from the surface of the body; and from dirt brought in from the streets on boots of the occupants. Certain bacteria, possessed of constant and well-defined characters, furnish means whereby pollution of each of these three kinds can be detected.

(1) *Pollution from material derived from the upper respiratory passages.* It has been shown (Gordon, *L.G.B.* 1902-3, ib. 1903-4) that certain streptococci, owing to their constant abundance in normal human saliva, provide a test whereby the presence of particles of that material may be identified, and that Streptococcus salivarius (see p. 143) in particular furnishes a means whereby 0·000,001 c.c. or less of saliva can be detected in air.

(2) *Pollution from material detached from the skin.* Owing to its constant abundance on the normal skin, Staphylococcus epidermidis albus forms a means whereby particles detached from the surface of the body may be detected (Gordon, *L.G.B.* 1904-5). The

characters of this staphylococcus have been stated before (p. 144).

(3) *Pollution by material brought in from the street on boots.* Material of this kind consists largely of horse-dung, and may be recognized by the presence of B. coli, spores of B. enteritidis sporogenes, and certain streptococci. B. mycoides is also not infrequently contained in such material, and thus serves as an additional bacterial index whereby its presence may be detected in air.

Particulate pollution of the three kinds mentioned above is most readily recognized by exposing plates filled with broth to the air, by then incubating this broth anaerobically for 48 hours at 37° C., and isolating and testing such index micro-organisms as may have developed in the broth by that time. Plates of solid media may also be exposed for the same purpose, and to this end solidified blood serum, ordinary agar, or the medium of Drigalski and Conradi may be exposed to the air.

Sewer Air.

Recent observations made by Andrewes and Hurtley (*Report on the sewer air of Hampstead,* 1905), and by Horrocks (*P.R.S.*, Feb. 7, 1907), show that by similar methods particles of sewage may under certain circumstances be detected in the air of sewers or drains. Horrocks has even succeeded by this means in demonstrating the presence of B. typhosus in the air of a drain through which typhoid excreta were passing (loc. cit.).

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